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Factors Influencing the Requirement for Polyunsaturated Fatty Acids

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THE purpose of this presentation is to discuss several factors or conditions which have an influence upon the requirement of animals for polyunsaturated acids, and which may influence the metabolism of these substances. No attempt is made to be comprehensive, and reference is made only to representative examples of the phenomena mentioned. For a more detailed discussion of essential fatty acid deficiency, and for more complete documentation of the general statements made in this presentation, the reader is referred to recent reviews on the subject.[1,2]

EFFECTS OF THE STRUCTURE OF POLYUNSATURATED ACIDS

The polyunsaturated fatty acids exist in several families, the members of which differ in total unsaturation and chain length but are alike in the position of the double bond nearest the terminal methyl group. Thus the linoleic acid family, known as essential fatty acids, has two or more *cis* double bonds, the first of which occurs at the sixth carbon atom from the methyl group. The oleic acid family has its first double bond at the ninth carbon atom.

Linoleic and related acids allow growth and maintenance of normal skin in rats and other species. The linolenate family allows growth but cannot cure the dermal symptoms of essential fatty acid deficiency. Oleate is synthesized by tissue and is not required in the diet. 5,8,11-Eicosatrienoic acid, of the oleate family, occurs in abnormally large amounts in essential fatty acid-deficient animals. It is thus clear that polyunsaturated acids are not all equal in biological activity. The metabolic function connected with maintenance of normal skin cannot be met by linolenate or oleate-type acids. Linoleate and arachidonate are the most abundant fully effective essential fatty acids. The former is found in plants and animals, the latter only in animal lipids. Arachidonate is more potent as an essential

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fatty acid than is linoleate from which it is derived metabolically. Only the all-*cis* forms of the unsaturated acids are effective as essential fatty acids. Recently many reports have indicated that polyunsaturated fatty acids have a hypocholesterolemic effect. At first this was assumed to be solely an essential fatty acid activity, but it is now known that oils rich in linolenate-type acids and poor in linoleate-type acids have an even stronger hypocholesterolemic effect than does linoleate.[3] Thus it appears that the transport function of polyunsaturated fatty acids can be met by both linoleate- and linolenate-type acids. The efficacy of the individual types of polyunsaturated fatty acids in meeting the requirement for reproduction or other functions has not been tested.

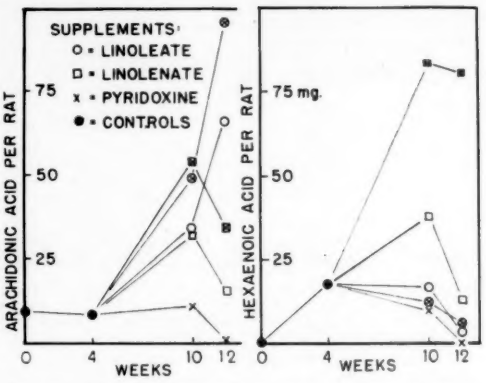

FIG. 1. Effect of pyridoxine upon synthesis of arachidonic and hexaenoic acids in the intact rat. From: WITTEN, P. W. and HOLMAN, R. T. *Arch. Biochem.*, 41: 266, 1952.[8]

THE EFFECT OF SEX

The essential fatty acids are known to be required for normal reproduction in animals. Severely deficient females are unable to conceive when mated with normal males, and deficient males are unable to sire litters with normal females. The reproductive organs of animals contain lipids in which the concentration of polyunsaturated fatty acids is extremely high. A fat-free diet fed to female rats during the gestation period causes the formation of defective young that survive only a few days. The requirement of essential fatty acids has been found to be higher for males than for females.[4] The fundamental cause of this difference is not yet determined.

THE EFFECT OF AGE

The older the animal when first fed a fat-free diet, the longer is the time required to develop essential fatty acid deficiency. In weanling (twenty-one day old) male rats fed a fat-free diet a severe deficiency develops in nine to twelve weeks. Adult rats are resistant to the development of deficiency. In one study it was necessary to reduce the animals to half weight and then feed the fat-free diet *ad libitum* to induce transitory symptoms of deficiency during the period of regrowth.[5] In another study, in adult rats fed an essential fatty acid-free diet *ad libitum* mild and transitory dermal symptoms developed at about thirty-five weeks[6] and stress of dietary cholesterol had little effect on adult rats. However, a stress of dietary cholesterol has an accelerating effect upon essential fatty acid deficiency which is greater in fifteen day old rats than in twenty-one day old rats.[7] The feeding of an essential fatty acid-free diet to pregnant female rats causes the young to be born with definite abnormalities. These several observations indicate that the younger an animal is when given a fat-free diet, the more readily is it depleted of essential fatty acid reserves.

THE EFFECT OF WATER BALANCE

Essential fatty acid deficiency increases the permeability of the skin, decreases capillary resistance, impairs renal function and increases water intake. The excessive evaporation of water from the skin probably contributes to the dry character of the dermatosis. The dermal symptoms of essential fatty acid deficiency can be accentuated by a dry atmosphere and minimized by a humid one. The onset of dermal symptoms can be hastened by restricting the water intake of rats fed a fat-free diet. Whether this really accentuates the deficiency or only the dermal difficulties is not known.

EFFECT OF PYRIDOXINE

Both pyridoxine and essential fatty acid deficiencies induce dermatites which are superficially somewhat similar, and the double

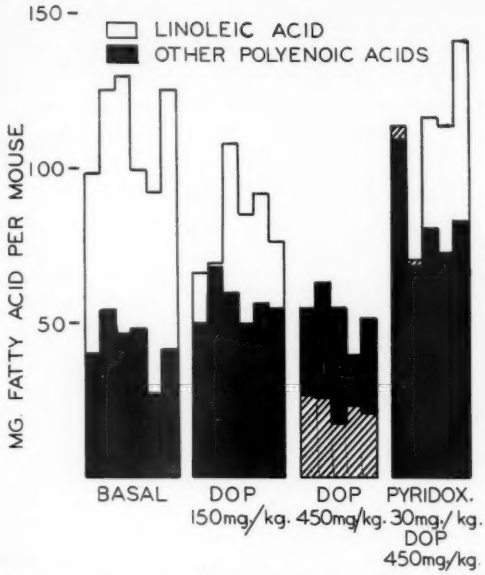

FIG. 2. Effect of desoxypyridoxine (DOP) and pyridoxine supplements upon polyunsaturated acids in intact mice. From: HOLMAN, R. T. Proceedings of the Second International Conference of Biochemical Problems of Lipids.[9]

deficiency of these nutrients induces severe deficiency more rapidly than either singly. Rats deficient in both pyridoxine and essential fatty acid were given supplements of pyridoxine, linoleate, linolenate, pyridoxine plus linoleate and pyridoxine plus linolenate.[8] The total tetraenoic, pentaenoic and hexaenoic acids in the carcass of the several groups of rats were determined by alkaline isomerization. The synthesis of tetraenoic acid from linoleate was found to be enhanced when pyridoxine was fed with linoleate and the synthesis of hexaenoic acid from linolenate was enhanced by pyridoxine (Fig. 1). Thus it appears that this vitamin is involved in the conversion of dietary linoleate and linolenate to the more highly unsaturated longer-chain acids in tissue.

Administration of the antimetabolite, desoxypyridoxine, to mice depressed the deposition of linoleate in their carcasses.[9] Supplementation of mice with pyridoxine as well as desoxypyridoxine restored the total body linoleate and increased the synthesis of polyenoic acids other than linoleate (Fig. 2). Recent preliminary results from our laboratory suggest that even the synthesis of eicosatrienoic acid is impaired when desoxypyridoxine is fed.

EFFECT OF DIABETES

In an attempt to accelerate essential fatty acid deficiency, a condition was sought in which fat catabolism is increased. Alloxan diabetes was induced in rats fed a fat-free diet, and the onset of dermal symptoms took place rapidly,[10] reaching, at one month, a stage of severity found in simple essential fatty acid deficiency only after three to four months. Stress of dietary cholesterol had no significant additional effect upon the deficient rats with diabetes. The diabetic subject is known to have hyperlipemia and hypercholesterolemia and excessive lipid transport may be the basis for the accelerated essential fatty acid deficiency.

THE EFFECT OF HYPOTHYROIDISM

Hypothyroidism is also accompanied by hypercholesterolemia. Therefore this abnormal metabolic condition was induced in rats fed a fat-free diet to determine if it also accelerates essential fatty acid deficiency. Thiouracil fed in a fat-free diet also accelerated essential fatty acid deficiency in weanling male rats.[11] Hypothyroidism increased the requirement for essential fatty acid so much that 1 per cent linoleate no longer fully protected the rats from essential fatty acid deficiency.

EFFECT OF HYPERCHOLESTEROLEMIC AGENTS

To further test the hypothesis that hypercholesterolemia and the attendant fat transport are the cause of accelerated essential fatty acid deficiency in diabetes and hypothyroidism, three hypercholesterolemic substances were tested. Dietary tetramethyl benzidine (0.2 per cent) induced accelerated symptoms of essential fatty acid deficiency in rats fed a fat-free diet without an appreciable hypercholesterolemia. Intraperitoneal injections of a nonionic detergent, triton, and intraperitoneal injections of an amino nucleoside derived from aureomycin also accelerated the essential fatty acid deficiency. Corn oil offered some protection only against the effects of the latter, which induced a profound hypercholesterol-

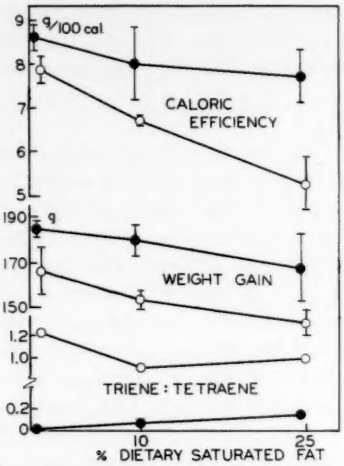

FIG. 3. Effects of dietary saturated fat upon essential fatty acid-deficient rats. From: PEIFER, J. J. and HOLMAN, R. T. *J. Nutrition*, 68: 155, 1959.[12]

emia. Dietary cholic acid, which is known to induce hypercholesterolemia, has been found to accelerate essential fatty acid deficiency as well.

EFFECT OF DIETARY CHOLESTEROL

Dietary cholesterol is known to induce hypercholesterolemia in animals, and the effect of this stress upon essential fatty acid deficiency has been studied in some detail.[7] Cholesterol (1 per cent) fed in a diet free of essential fatty acids hastens the appearance of dermal symptoms of essential fatty acid deficiency and the effect is more regular and severe with fifteen day old male rats than with twenty-one day old weanlings. Linoleate fed with the cholesterol either prevents or cures the deficiency, indicating that dietary cholesterol is no stress if intake of essential fatty acids is adequate. Dermal symptoms of deficiency continue to become more severe even after cholesterol is withdrawn from the diet, indicating that once the accelerating effect of cholesterol had begun depletion of the animal, continued stress of cholesterol is not needed to maintain the deficiency. That feeding cholesterol accentuates essential fatty acid deficiency is evidenced by a lower food efficiency, a lower weight gain, retardation of testicular development and an accentuated pattern of polyunsaturated fatty acids in heart lipids characteristic of essential fatty acid deficiency. The ratio of trienoic to tetraenoic acids in heart lipids, which is normally of the order of <0.4, was 1.8 for rats fed an essential fatty acid-free diet. Dietary cholesterol in a diet free of essential fatty acids raised this ratio to 2.4, indicating a more severe essential fatty acid deficiency. When cholesterol was fed with linoleate, no increase was observed. Thus objective chemical evidence indicated an intensified essential fatty acid deficiency. This indicates that dietary cholesterol increases the requirement for essential fatty acids, but no quantitative evidence on this point is at hand.

EFFECT OF DIETARY SATURATED FAT

The influence of saturated fat upon essential fatty acid metabolism has been recently studied from several aspects in our laboratory.[12] Six groups of weanling male rats were fed two sets of diets. In these diets, essential fatty acid was lacking, and the content of hydrogenated coconut oil (fully saturated fat) was 1, 10 or 25 per cent. In the other three diets, 0.5 per cent ethyl linoleate was incorporated and the content of hydrogenated coconut oil was 0.5, 9.5 or 24.5 per cent. Weight gain and caloric efficiency were determined upon all groups. After seven weeks, the animals were sacrificed, and the polyunsaturated fatty acid content of the heart tissue was determined. The data are summarized in Figure 3.

Caloric efficiency decreased as the content of saturated fat increased in the presence or absence of essential fatty acids in the diet. The caloric efficiency decreased more drastically in the absence of essential fatty acids. Weight gain decreased as the content of saturated fat in the diet increased, whether or not essential fatty acids were fed. The chemical index of essential fatty acid deficiency, trienoic acid:tetraenoic acid ratio, likewise indicated that high levels of saturated fat imposed a metabolic strain. The ratio was high and rather constant in all animals that were not fed essential fatty acids. However, the normally low ratio induced when essential fatty acids were fed rose significantly when higher levels of saturated fat were fed. These three criteria indicate that the require-

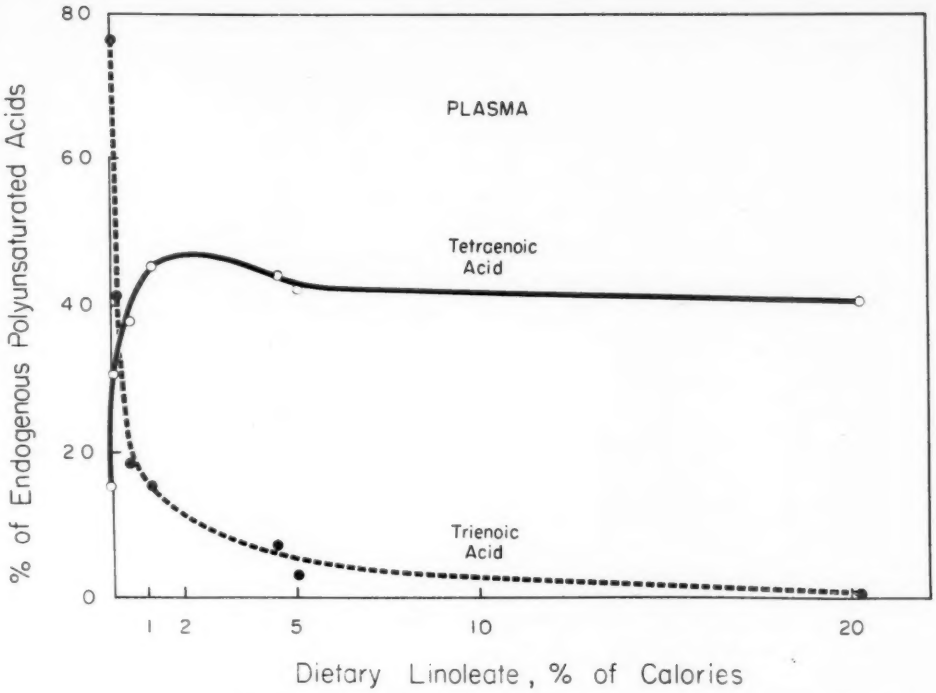

FIG. 4. Relationships between dietary linoleate and the trienoic and tetraenoic acids of plasma of rats.

ment for essential fatty acids is increased when saturated fat is fed at higher levels. That is, the requirement of essential fatty acids is not a fixed amount per day but is proportional to the quantity of non-essential fat in the diet.

In the previous paragraphs, several influences have been shown to increase the requirement for essential fatty acids. These influences have in common their hyperlipemic effect. In some cases the hyperlipemic effect may be only of secondary significance because the major components of plasma lipids usually rise and fall together. The current emphasis on cholesterol content may be a misplaced emphasis dictated by the relative ease with which cholesterol can be measured, and the difficulty of measuring other lipid components. The hyperlipemia induced by the increased transport of saturated fat or cholesterol also involves an increased transport of polyunsaturated fatty acids as components of cholesterol esters, phospholipids and triglycerides. If the polyunsaturated fatty acid is not provided from dietary sources, it must be mobilized from stores and from tissues, for these substances are not synthesized except from required dietary precursors. If mobilization of polyunsaturated fatty acids from tissues is sustained, the tissues will be depleted and deficiency will result. Thus prolonged intake of high levels of fat containing insufficient essential fatty acids, or sustained hyperlipemia from other causes, may produce chronic or marginal essential fatty acid deficiency.

EFFECT OF PROPORTION OF ESSENTIAL FATTY ACID CALORIES

The observation that increasing the dietary saturated fat increases the requirement for essential fatty acids indicates that this requirement is a relative matter. This prompted a practical test designed to evaluate two common dietary fats for their essential fatty acid efficacy at levels of 10 and 40 per cent of calories, corresponding to the approximate proportions in which butterfat and total fat occur in the American diet. Seven groups

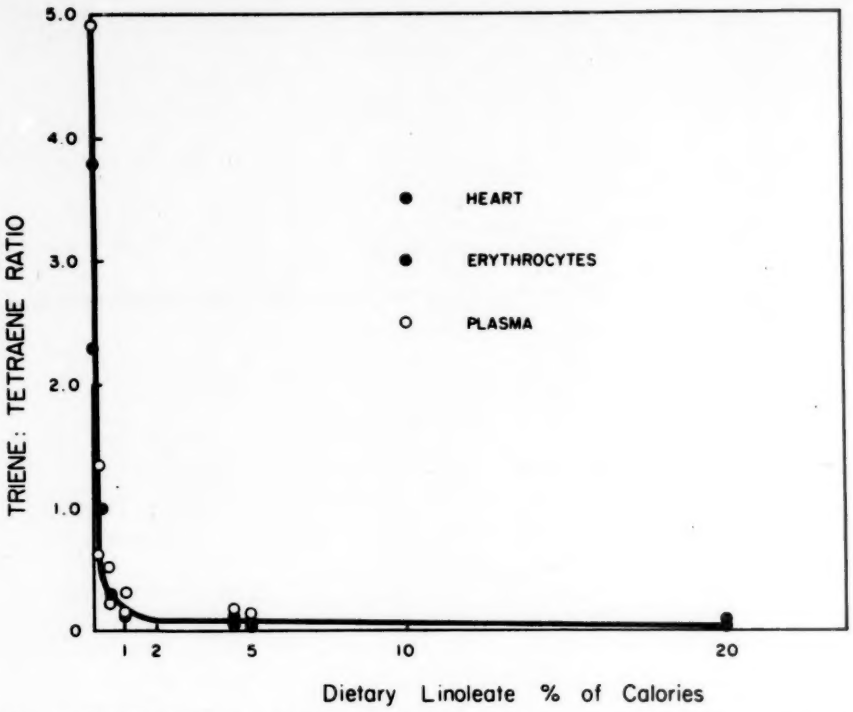

FIG. 5. Relationship between trienoic acid : tetraenoic acid ratios of rat heart, erythrocytes and plasma in dietary linoleate. From: HOLMAN, R. T. *J. Nutrition*, 70:405, 1960.[13]

of male weanling rats were fed synthetic diets containing the following proportions of fat: (1) none, (2) 10 per cent of calories as butterfat, (3) 10 per cent of calories as butterfat : cottonseed oil (4:1), (4) 10 per cent of calories as cottonseed oil, (5) 40 per cent of calories as butterfat, (6) 40 per cent of calories as butterfat : cottonseed oil (4:1), and (7) 40 per cent of calories as cottonseed oil. After eighty-nine days the rats were sacrificed and the polyunsaturated fatty acids were determined in the lipids of plasma, erythrocytes and heart tissue.[13] When the tetraenoic acid content of the endogenous polyunsaturated fatty acid of plasma was plotted against the dietary linoleate expressed as per cent of calories, a curve was obtained having a sharp break near 1 per cent of calories (Fig. 4). As dietary linoleate was increased, the proportion of trienoic acid in the endogenous polyunsaturated fatty acids decreased precipitously and remained relatively constant above 1 per cent of calories. Similar plots made of data from erythrocytes and heart tissue gave essentially the same curves. When the ratio of trienoic acid to tetraenoic acid is plotted against dietary linoleate, a hyperbolic curve is obtained in which an abrupt change in slope occurs near 1 per cent of calories as linoleate (Fig. 5). The curves for data from plasma erythrocytes and heart tissue are superimposable, indicating that the metabolic lesion in essential fatty acid deficiency is reflected in these three tissues and perhaps in all tissues.

In Figure 5, the horizontal leg of the hyperbola represents a normal pattern of polyunsaturated fatty acids and the vertical leg represents an abnormal condition. The point of maximum change in slope represents the minimum requirement for essential fatty acids, 1 per cent of calories as linoleate. This value, being determined by an objective chemical evaluation of changes in composition of tissue, is unaffected by subjective evaluations such as grading dermal symptoms. The evaluation

TABLE I

Correlation Coefficients Between Dietary Linoleate and Individual Heart Tissue Polyenoic Acids

Dienoic acid	0.88
Trienoic acid	−0.44
Tetraenoic acid	0.70
Pentaenoic acid	0.93
Hexaenoic acid	−0.37

NOTE: At 1 per cent level R = 0.37.

of essential fatty acid requirements by this means is more specific than is measurement of body weight which is a reflection of many influences. The data used for determination of essential fatty acid requirements were derived from fifty-one rats fed seven different diets, involving three levels of dietary fat and three kinds of dietary fat. Even so, the relationship between triene:tetraene ratio and dietary linoleate is unmistakable.

The data obtained from the heart lipids have been treated statistically using a Univac 1103 digital computer, to obtain correlation coefficients between dietary linoleate and the individual heart polyenoic acid types, plus a regression equation relating dietary linoleate to heart polyenes. The correlation between these variables is given in Table I.

These data indicate that of the individual tissue polyenes, pentaenoic acid bears the most direct relationship to dietary linoleate. A plot of pentaenoic acid versus dietary linoleate, given in Figure 6, indicates pentaene to be a reasonable measure of dietary linoleate, but that at low levels of linoleate the function is curvilinear.

The derived regression equation for heart samples relating dietary linoleate to the several variables, and in which the function is more linear, is the following:

$$\begin{aligned}\text{Calc. per cent dietary linoleate} = &-5.086 \\ &+ 0.04264\ (\text{dienoic acid}) \\ &+ 0.01647\ (\text{trienoic acid}) \\ &- 0.02190\ (\text{tetraenoic acid}) \\ &+ 0.07444\ (\text{pentaenoic acid}) \\ &+ 0.00602\ (\text{hexaenoic acid})\end{aligned}$$

where values for heart fatty acids are expressed as mg./100 gm.

Values for dietary linoleate calculated from this equation are plotted against the values

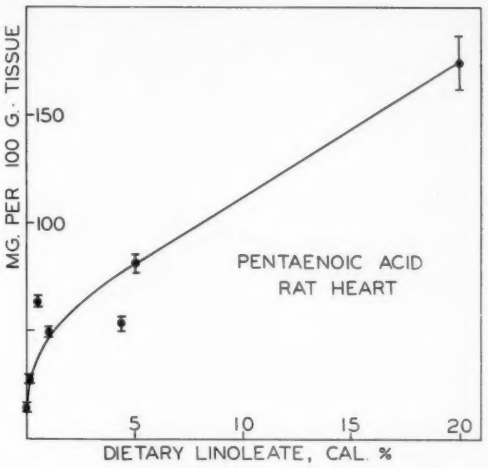

FIG. 6. Relationship between heart pentaenoic acid and dietary linoleate in a population of fifty-one rats fed seven different diets.

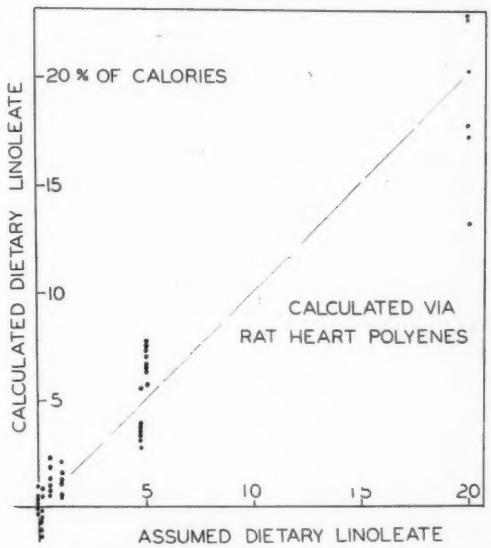

FIG. 7. Comparison of estimated dietary linoleate and values calculated via the regression equation.

predicted from diet composition in Figure 7. The standard deviation between measured and calculated values is 1.89 per cent of calories. This magnitude of error is thus low enough so that the calculation of dietary linoleate from tissue polyunsaturated fatty acids may be used for assessment of the dietary status of essential

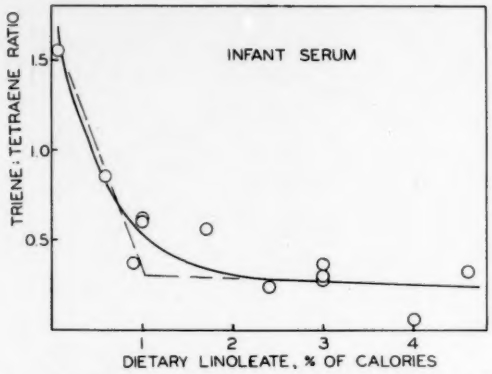

FIG. 8. Relationship between dietary linoleate and serum trienoic acid:tetraenoic acid ratio in infants. Calculated from data of WIESE, H. F., HANSEN, A. E. and ADAMS, D. J. D. *J. Nutrition*, 66: 345, 1958.[14]

fatty acids. It should be emphasized that the dietary linoleate was calculated from chemical analyses alone. Other factors which may be included in the calculation to increase the accuracy of prediction are being studied.

This type of determination of essential fatty acid requirement is applicable to human beings. An example of its potential is illustrated in Figure 8 where limited data from the literature have allowed the relating of trienoic acid:tetraenoic acid ratio in infant serum to dietary linoleate.[14] The ratio has been calculated for a series of samples including all the infants who were given diets in which the fat existed as triglyceride. It will be seen that a curve is obtained resembling that obtained from analyses of rats. From the curve the minimum requirement of infants for linoleate is estimated to be 1 per cent, agreeing with the original authors who arrived at this value by a more difficult evaluation of the data. Lack of data for pentaenoic and hexaenoic acids precluded calculation of a predictive equation for dietary essential fatty acids in this case.

A method is now available for an objective measure of the requirement of man or animals for essential fatty acids at any age and in any physiologic condition. From similar data on human subjects, it may also be possible to assess the status of their essential fatty acids and to correct their diets if necessary.

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Regulation of Cholesterol Biosynthesis and Catabolism

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IT IS now fairly well established that the liver is a major site of cholesterol biosynthesis and degradation. These complex processes are, of course, interrelated, but for purposes of clarity they will be considered separately here.

BIOSYNTHESIS

Since the demonstration by Bloch and Rittenberg[1] in 1942 that a two-carbon unit, acetate, could provide all the carbon atoms necessary for cholesterol biosynthesis, most steps in the biosynthetic pathway have been elucidated. Two principal features of this work were the demonstrations of the seemingly obligatory roles of mevalonic acid ($\beta\delta$-dihydroxy-β-methylvaleric acid)[2] and squalene.[3] The generally accepted scheme of cholesterol biosynthesis from acetate is outlined in Figure 1, although a number of the intermediate compounds have not yet been fully identified.

Even when less was known of the details of cholesterol biosynthesis, a number of investigators had begun to study some of the factors which affect this process. Today it is possible to assign the action of some of the known stimulatory and inhibitory factors to specific sites in the biosynthetic scheme.

Diet

The effects of diet were among the earliest studied. In the intact, fasting rat there is a progressive decrease in biosynthetic capacity, slightly subnormal synthesis being observed after twenty-four hours, a 50 per cent decrease after forty-eight hours, and a 67 per cent decrease in three days.[4] However, liver slices from rats fasted for twenty-four hours show a more marked drop in cholesterogenesis.[5] Starvation is actually not necessary to show this effect, for even restriction of caloric intake will inhibit hepatic cholesterogenesis.[5,6]

In a complete diet, the presence of cholesterol or one of its precursors will impair liver biogenesis of this sterol. Gould and associates[7,8] demonstrated that dietary cholesterol profoundly affected the ability of dogs or rabbits to convert acetate to cholesterol. Tomkins, Sheppard and Chaikoff[9] showed the same situation to be true in the rat (Table I). In rats fed a 2 per cent cholesterol diet for two weeks, hepatic synthesis was depressed 75 to 90 per cent.[10] In rabbits fed cholesterol over longer periods, the endogenous contribution to the circulating cholesterol appeared to be only 5 to 10 per cent.[11] Feeding mevalonic acid,[12] squalene, lathosterol (Δ^7-cholesterol) or 7-dehydrocholesterol[13,14] to rats causes inhibition of cholesterogenesis in the liver (Table II). Lathosterol has been shown to be converted to cholesterol.[15] Cholesterol and 7-dehydrocholesterol seem to be interconvertible in the intestine.[16] The atherogenicity of 7-dehydrocholesterol has

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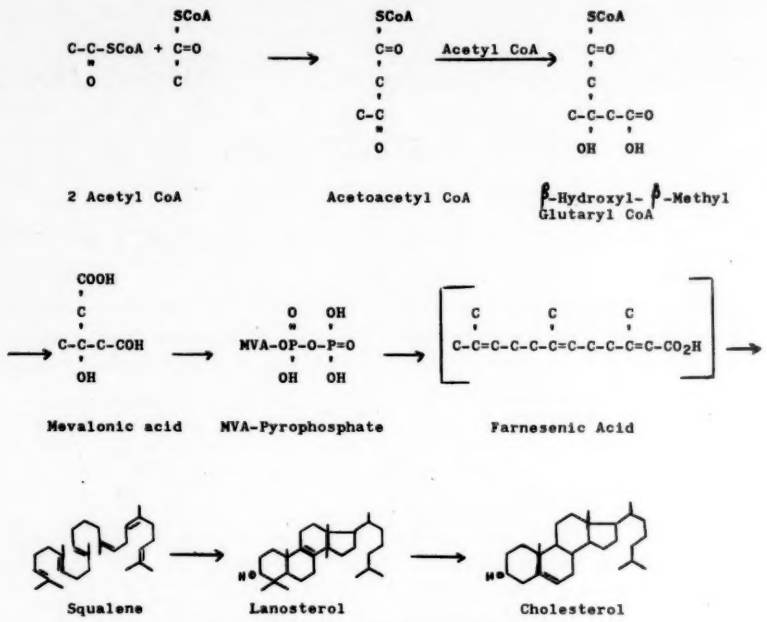

FIG. 1. Pathway of cholesterol biosynthesis.

been attributed to its rapid conversion into cholesterol.[17] Feeding lanosterol does not seem to affect hepatic cholesterogenesis, however.[13]

The effect of dietary fat on cholesterol biosynthesis has also been studied, especially with the recent emphasis on the role of unsaturated fat as a hypocholesterolemic agent. Alfin-Slater and co-workers[18] have reported that, in general, a high fat diet does not affect hepatic cholesterol synthesis. Opinion is divided on the comparative effects of diets containing saturated fat versus those containing unsaturated fat. Three groups of investigators[19–21] found that unsaturated fat stimulated

TABLE I
Effect of Fasting and of Cholesterol Feeding on Cholesterol Synthesis

Species	No.	Diet	Cholesterol Synthesis	Reference
Rat	3	Normal	1.00	5
	5	Fasted, 24 hr.	0.08	5
	3	Fasted, 48 hr.	0.17	5
	4	Fasted, 72 hr.	0.01	5
Dog	2	Normal	1.00	8
	2	Cholesterol (1%)	0.06	8
Rat	2	Normal	1.00	9
	9	Cholesterol (5%)	0.03	9
Rat	6	Normal	1.00	24
	6	Cholesterol (1%)	0.07	24

TABLE II
Effect of Steroid Feeding on Cholesterol Synthesis in the Rat

Compound Fed	No.	Cholesterol Synthesis	Reference
None	7	1.00	13
Squalene	2	0.36	13
Lathosterol	4	0.78	13
7-Dehydrocholesterol	4	0.74	13
Coprosterol	1	0.32	13
Ergosterol	1	0.71	13
None	8	1.00	14
Cholestenone	5	0.03	14
Dehydroisoandrosterone	5	0.14	14
7-Dehydrocholesterol	3	0.16	14
Lathosterol	2	0.08	14

TABLE III
Type of Dietary Fat and Cholesterol Synthesis in the Rat

Dietary Fat							Reference
Normal	Corn Oil	Rapeseed Oil	Cottonseed Oil	Coconut Oil	Hydrogenated Cottonseed Oil	Lard	
...	1.00	...	...	0.07	...	...	19
1.00	1.68	1.70	...	0.66	...	...	20
1.00	1.01	...	...	0.90	...	...	21
...	1.00	...	1.43	...	1.36	1.44	22
...	1.00	...	...	...	...	0.76	23

cholesterol biosynthesis more than did saturated fat. Two other groups[22,23] report no such differences (Table III). Fat-free diets will also inhibit cholesterogenesis.[19,22] The data suggest that accumulation of cholesterol in the liver causes a decrease in cholesterol biosynthesis, at least in this one tissue. Extrahepatic biosynthesis may not be affected to the same degree.

Alfin-Slater et al.[18] suggested that cholesterol levels in the liver might control cholesterol biosynthesis. Frantz and co-workers[24] proposed an inverse relation between total concentration of cholesterol in liver slices of the rat and the logarithm of the rate of hepatic biosynthesis. Gould,[25] however, believes that this suggestion is probably an oversimplification. In cholesterol-fed rats he found an inverse linear relation between the amount of free cholesterol in the liver and the logarithm of the biosynthetic rate in this tissue. This seems more logical in view of Bucher and McGarrahan's demonstration[26] that the enzymes required for cholesterol biosynthesis in the liver are found principally in certain particulate fractions which contain cholesterol in the free, i.e., not esterified, form. Swell and co-workers[27] demonstrated that cholesterol biosynthesis is sensitive to fluctuations in the supply of exogenous cholesterol. The biosynthesis of cholesterol from acetate-1-C^{14} in lymph-fistula rats was much greater (700 per cent) than in normal rats, although liver and plasma cholesterol levels were about the same in both.

Not only are liver homogenates from starved rats incapable of synthesizing cholesterol, but when added to normal homogenates they also inhibit biosynthesis.[28] The site of the starvation block appears to be between synthesis of hydroxymethylglutarate and formation of squalene.[29] Bucher and co-workers[30] have investigated the biosynthesis of cholesterol in the livers of fasting and cholesterol-fed rats using acetate, mevalonate or squalene as radioactive substrates. They find that incorporation of mevalonate was not influenced greatly by starvation. Conditions of starvation, therefore, primarily affect some phase of the biosynthetic pathway preceding utilization of mevalonic acid. In experiments on biosynthesis with liver homogenates prepared from cholesterol-fed rats, Gould and Popjak[31] also found that the block in biosynthesis of cholesterol preceded formation of mevalonate.

Hormones

Among other physiologic factors regulating cholesterol biosynthesis are thyroid hormones, estrogens and pituitary hormones. Cholesterol biosynthesis is reduced during pregnancy[32] and by administration of estrogens,[33] but little work has been performed on their precise mode of action.

The hypothyroid state appears to be associated with a general slowing of all metabolic processes, including synthesis of cholesterol.[34] Cholesterol biosynthesis is accelerated in hyperthyroid states and decreased in hypothyroid conditions.[35–37] Since Schettler[38] has shown that total body cholesterol also changes under the influence of the thyroid, we might expect that the increase or decrease of liver choles-

TABLE IV
Effect of Thyroid Hormone on Cholesterol Synthesis

Species	Normal	Hyperthyroid	Hypothyroid	Reference
Man	1.00	—	0.16	39
Rat	1.00	...	0.60	37
Rat	1.00	2.50	0.82	40
Rabbit	1.00	1.00	0.75	40
Guinea pig	1.00	—	0.50	40
Rat	1.00	2.03	0.47	43

terol affects hepatic biosynthesis of this sterol. The most dramatic manifestation of hypothyroidism inhibiting cholesterol biosynthesis has been observed in man[39] (Table IV).

Fletcher and Myant[40] observed that in hyperthyroid rats there is an increase in cholesterol synthesis due to acetate but not due to mevalonate. In hypothyroid rats incorporation of acetate into cholesterol is depressed, but that of mevalonate is not. Again, a step preceding utilization of mevalonate in cholesterol biosynthesis is affected. The rate of formation of mevalonate thus appears to be a "pacemaker" for the entire biosynthetic sequence.

The effect of thyroid hormones upon excretion of bile and bile acids is an important factor. Friedman and co-workers[41–43] have demonstrated that there is an increased output of bile and of biliary cholesterol in hyperthyroid rats. Conversely, hypothyroid rats excreted less biliary cholesterol. These effects on output of bile have been confirmed[44,45] and an increased excretion of bile salts has also been noted in hyperthyroidism.

Radiation

Radiation with x-rays causes increased hepatic cholesterol biosynthesis[46,47] associated with reduction of liver cholesterol levels[47,48] (Table V). Further analysis reveals that incorporation of acetate-C^{14} into cholesterol is stimulated to a greater extent by radiation than is the incorporation of mevalonate[25,46] (Table VI). Radiation will stimulate cholesterol synthesis even in hypophysectomized rats.[49] Evidently, the effect of radiation is not dependent upon the pituitary gland.

TABLE V
Effect of Radiation on Cholesterol Synthesis from Acetate-C^{14}

Normal	Radiated (dose)	Reference
1.00	1.95 (2,500 r)	46
1.00	4.20 (3,750 r)	46
1.00	3.95 (5,000 r)	46
1.00	3.30 (300 r)	47
1.00	7.50 (900 r)	47
1.00	23.20 (2,400 r)	47
1.00	1.40 (400 r)	48

Glycolysis

The effect of glycolysis upon cholesterol biosynthesis must also be noted. Bucher and McGarrahan[26] have shown that the biosynthetic capacity of liver homogenates of the normal rat could be abolished by removal of glycogen and restored by the action of hexose diphosphate. Siperstein[50,51] has shown that reduced triphosphopyridine nucleotide, generated by oxidation of glucose through the hexosemonophosphate pathway, is required for cholesterol biosynthesis. The lowered cholesterol biosynthesis in fasted rats may also be due, in part, to lowered glycogen levels. Radiation causes large increases in liver glycogen levels of fasted rats[52] but not in normally fed rats.[47] The extremely high rates of cholesterol biosynthesis in rats exposed to radiation suggest that the liver glycogen level is only one of several factors causing increased synthesis. Elevation of liver glycogen levels, however, may explain the stimulatory effect of corticotropin[32] on cholesterol biosynthesis. Depression of cholesterogenesis by hypophysectomy[53] might likewise be attributed to lowering of glycogen levels, since

TABLE VI
Factors Affecting Cholesterol Synthesis from Acetate and from Mevalonate

Effect	Normal	Acetate-C^{14}	Mevalonate-C^{14}	Reference
Fasting	1.00	0.02	0.17	30
Cholesterol feeding	1.00	0.006	0.18	30
Thyroid	1.00	2.50	1.00	40
Radiation	1.00	5.60	2.00	30
Radiation	1.00	4.79	4.23	46

TABLE VII
Factors Influencing Cholesterol Biosynthesis

Increase	Decrease
Thyroid hormones	Cholesterol feeding
Radiation	Starvation
Triton WR-1339	Thyroidectomy
Mn^{++}, Fe^{+++}	V^{+++++}

prefeeding a diet high in glucose or starch to hypophysectomized rats will restore hepatic cholesterogenesis to normal.[54]

Other Factors

A few external, or pharmacologic, factors which influence cholesterol biosynthesis should also be mentioned. Injection of Triton WR-1339 (a polymer of p-isooctylpolyoxyethylphenol) markedly stimulates cholesterol biosynthesis[55] in rats. The biosynthetic step primarily affected here also appears to be that between acetate and mevalonate.[30]

Curran[56,57] investigated the role played by certain metal ions in cholesterol biosynthesis by the liver of the rat. He found that chromous, chromic, manganous and ferric ions each stimulated biosynthesis, whereas vanadous, vanadic, ferrous and cobaltous ions each depressed biosynthesis. The effect of vanadium is exerted between the synthesis of hydroxymethylglutarate and of squalene.[58]

Since the initial reports by Cottet and coworkers[59,60] concerning the hypocholesterolemic action of derivatives of 2-phenylbutyric acid, it has been demonstrated that these compounds and many of their analogs[61–63] all inhibit the acetylation of coenzyme A. Goldstein[64] has found that most phenyl-substituted, short-chain fatty acids will inhibit cholesterol biosynthesis.

A compound commonly designated as MER-29 (1 - [4 - diethyl - aminoethoxy]phenyl - 1 - [p - tolyl] - 2 - [p - chlorophenyl]ethanol) (triparanol) has recently been reported to inhibit cholesterol biosynthesis in intact rats and monkeys.[65] In rats maintained on this drug and then given acetate-C^{14} there is no diminution of digitonin-precipitable radioactivity but a marked decrease in the amount of cholesterol in the digitonide.[66] It was concluded that cholesterol biosynthesis is inhibited at some stage following cyclization of squalene. This non-cholesterol digitonin-precipitable material is now known to be desmosterol (24-dehydrocholesterol).[67]

In summary, the biosynthesis of cholesterol by the liver is under a type of homeostatic control, influenced by changes in the levels of free cholesterol and/or glycogen in the liver, and by withholding of the appropriate coenzymes (Table VII).

Frantz[68] suggested that the homeostatic control mechanism may depend on the presence of "active" cholesterol, as opposed to "inactive" cholesterol bound in lipoproteins or elsewhere. Thus, excess dietary cholesterol, for instance, would saturate all available binding sites and result in an excess of "active" cholesterol, which then inhibits biosynthesis. On the other hand, x-radiation could promote formation of cholesterol-binding lipoproteins (receptor molecules) in the liver, thereby promoting biosynthesis. Triton WR-1339 might likewise enhance removal of active cholesterol by complex formation, thus explaining its accelerating effect on cholesterol biosynthesis. Most of the inhibitors mentioned have in common some action on a stage in the biosynthetic pathway preceding formation of mevalonate.

DEGRADATION

Cholesterol is metabolized to bile acids, sex hormones and adrenocortical hormones (Fig. 2). Of these, only the formation of bile acid need be considered here since it has been estimated that from 70 to 90 per cent of the cholesterol synthesized daily is converted to bile acids.[69,70]

Bile acids enter into the enterohepatic circulation but are continuously lost in the feces by incomplete resorption through the intestine. Cholesterol may also be removed from the body by direct elimination. It is excreted directly into the bile; it then enters into the enterohepatic circulation together with the bile salts. What is not reabsorbed from the intestine is eliminated in the feces. If this reabsorption process is inhibited, e.g., by feeding roughage,[71] the contributions of the biliary pathway for re-

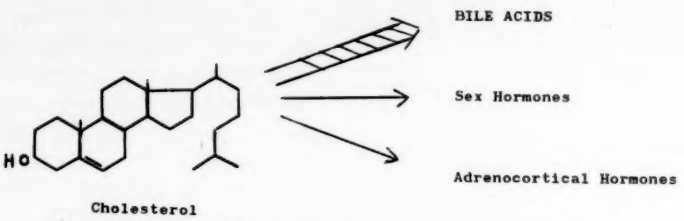

FIG. 2. Major pathways of cholesterol degradation.

moval of cholesterol as such will consequently be increased. Cholesterol is also excreted, probably as a process of desquamation of the epithelial cells, through the skin and into the intestinal tract. Quantitatively, these pathways of direct elimination are less significant for disposal of cholesterol in mammals than formation of hepatic bile acid.

The first conversion of cholic acid was demonstrated by Bloch, Berg and Rittenberg[72] in 1943 with rats fed deuterium-labeled cholesterol. For conversion of cholesterol to cholic acid (the principal bile acid found in man) the body must oxidize the side chain with removal of three carbon atoms, hydrogenate the double bond, invert the hydroxyl group at carbon atom 3, and introduce other hydroxyl groups at carbon atoms 7 and 12. Bergström and colleagues[73] have shown that hydroxylation of the nucleus in all probability precedes oxidation of the side chain.

We have already observed that the amount of free cholesterol in the liver affects cholesterol synthesis *de novo*. We might therefore presume that cholesterol degradation is similarly governed by the concentration of the major degradation products in the liver (feedback effect). Although there are less data available to justify such an assumption, the experimental evidence for it seems no less convincing.

Biliary Excretion

Eriksson[74] measured excretion of bile salts, in hyper-, hypo-, and euthyroid bile-fistula rats. He found the least excretion in the hypothyroid rats and most in the hyperthyroid rats. The extent of excretion of bile salts is directly related to cholesterol synthesis in these conditions. The intestinal flora are known to play an important role in the metabolism of bile acids, being responsible for scission of the bile acid conjugates[75] and for production of deoxycholic acid from cholic acid.[76] The half-life of bile acids in germ-free rats is seven days, but in normal (germ-full) rats it is only 1.8 days.[75] The germ-free animals have higher serum cholesterol levels.[77] Bergström and Danielsson[78] demonstrated that cholesterol oxidation *in vivo* is controlled via a feedback mechanism. Injection of sodium taurochenodeoxycholate caused decreased excretion of cholic acid in a bile-fistula rat. Thus, the concentration of bile acids supplied to the liver via the portal circulation influences the rate of synthesis of bile acids by the liver. In other words, cholesterol degradation is affected by bile acid concentrations.

This conclusion was also reached by Pihl[79] after he studied effects of bile acids on cholesterol absorption, deposition and synthesis in rats.

Other workers[80–83] have demonstrated that feeding bile acids or bile salts may raise serum cholesterol levels and inhibit hepatic cholesterol biosynthesis, presumably by inhibiting further cholesterol catabolism and formation of bile acid.

Using a mitochondrial preparation of the rat's liver which oxidizes cholesterol[84] Whitehouse and Staple have shown that mitochondrial preparations from bile-fistula rats (i.e., drained of circulating bile salts) oxidize cholesterol to a greater extent than do control animals.[85] They also found that addition of tauro- or glycocholic acids selectively inhibits oxidation of cholesterol, but not other substrates, by this system. These conjugates had a much smaller effect on the oxidation of 3α-, 7α-, 12α-trihydroxycoprostane (Table VIII), a finding which supports Bergström's hypoth-

TABLE VIII

Oxidation of Cholesterol and Trihydroxycoprostane (THC) by Liver Mitochondria from Normal and Bile-Fistula Rats and Inhibition by Added Bile Salts*

	Experiment 1		Experiment 2	
	Normal	Fistula	Normal	Fistula
Per cent cholesterol oxidation	10.7	25.9	7.3	10.2
Per cent inhibition of oxidation		Cholic	Tauro-cholic	Glyco-cholic
Cholesterol		81.5	45.5	49.7
THC		65.7	27.0	28.0

* From: WHITEHOUSE, M. W. and STAPLE, E. *Proc. Soc. Exper. Biol. & Med.*, 101: 439, 1959.[85]

TABLE IX

Oxidation of Cholesterol-26-C^{14} by Mitochondria of Rat's Liver*†

Sex	Experiment No.	Per Cent of Oxidation‡		
		Diet		
		Normal	Unsaturated	Saturated
Mixed	1	15.6	1.7	9.6
	2	15.2	17.4	17.6
	3	21.4	3.0	15.5
Females	1	5.3	6.6	15.2
	2	4.2	3.3	12.0
	3	21.0	1.9	10.4
	4	1.3	22.4	31.8
	5	11.7	2.9	4.5
Males	1	7.2	4.9	7.9
	2	15.1	13.9	23.7
	3	15.0	3.0	20.3
	4	11.1	2.8	11.2

* From: KRITCHEVSKY, D., KOLMAN, R. R., WHITEHOUSE, M. W., COTTRELL, M. C. and STAPLE, E. *J. Lipid Res.*, 1: 83, 1959.[90]

† All values corrected for equivalent amounts of mitochondria (mg. N).

‡ Computed as $BaC^{14}O_3$/cholesterol-26-C^{14}.

esis[86] that the rate-determining and feedback-sensitive step in formation of bile acids is probably nuclear hydroxylation, especially at the 7α- position.

The natural mechanisms regulating cholesterol biosynthesis and metabolism might therefore be represented after the manner of Gould[25] (Fig. 3). The rate-controlling step for cholesterol degradation is probably 7α-hydroxylation. For cholesterol biosynthesis the rate-controlling step lies between acetyl CoA and mevalonate.

The hypocholesterolemic effect of a diet rich in unsaturated fat has been attributed to increased excretion of fecal bile acid.[87,88] Lewis[89] has shown increased bile acid secretion in bile-fistula human beings under the influence of unsaturated fat. From the scheme presented, it would appear that cholesterol biosynthesis should be increased under these conditions. This has been shown to be so in rats.[19–21] Presumably, catabolism under these conditions far exceeds synthesis.

Using the mitochondrial cholesterol oxidase system of the liver described,[84] we have found that mitochondria from the livers of rats fed unsaturated fat oxidize cholesterol to a lesser extent than do mitochondria from rats fed relatively saturated fat[90] (Table IX). Wilson and Siperstein[91] showed that, when given a single tracer dose of radioactive cholesterol, rats fed lard excrete more radioactivity as bile acid than do rats maintained on corn oil. Excretion of neutral steroids and of total radioactivity was larger in the latter group, however.

Nicotinic Acid

This *in vitro* system has also been used to study the effects of feeding nicotinic acid.[92] We find a marked enhancement of cholesterol oxidation in rats fed nicotinic acid. This finding supports the initial suggestion of Altschul[93] that the mechanism of the hypocholesterolemic action of nicotinic acid involves enhancement of cholesterol catabolism (Tables X and XI). Of particular nutritional significance is the fact that nicotinamide, the usual form in which nicotinic acid is supplied

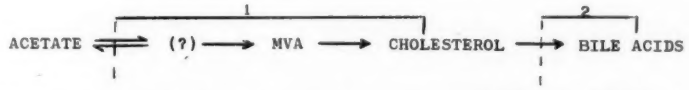

FIG. 3. Schematic diagram suggesting control mechanisms of cholesterol metabolism. 1, Feedback mechanism sensitive to liver cholesterol concentration. 2, Feedback mechanism sensitive to bile acid concentration.

TABLE X

Per Cent Oxidation of Cholesterol-26-C^{14} and Sodium Pyruvate-2-C^{14} by Mitochondria of Liver of Rats Given Nicotinic Acid (12 mg./day) in Their Drinking Water*

Experiment no.	Drinking Water	Per Cent Oxidation of Substrates†			
		Males		Females	
		Cholesterol	Pyruvate	Cholesterol	Pyruvate
1	Nicotinic	25.8	8.3	lost	lost
	Control	8.8	1.4	5.2	9.2
2	Nicotinic	6.6	5.2	19.3	22.1
	Control	6.0	9.2	26.8	31.6
3	Nicotinic	22.2	20.3	14.7	14.0
	Control	14.0	14.5	18.6	21.7

* From: KRITCHEVSKY, D., WHITEHOUSE, M. W. and STAPLE, E. *J. Lipid Res.*, 1:154, 1960.[92]
† All values are corrected for equivalent amounts of mitochondria (mg. N).

as a vitamin supplement, both *in vivo* and *in vitro* has little or none of the efficacy of nicotinic acid in promoting hypocholesterolemia and hepatic oxidation of cholesterol.

Metal Ions

We have also investigated the effects of metal ions on oxidation of cholesterol by mitochondria of the rat's liver.[94] Mitochondria from the livers of rats fed ferric, cobalt or nickel ions oxidized cholesterol to a greater extent than did preparations from control animals (Table XII). Our results suggest that this effect is due to enrichment of natural cofactors normally present in the particle-free supernatant fractions of the liver, which stimulate oxidation of cholesterol. Ferric ions appear to be required for hydroxylation of the cholesterol nucleus.[95]

Another consequence of feeding these metal ions is a possible reduction in the circulation of bile salts through the enterohepatic circulation. This would stimulate cholesterol oxidation by the liver. Siperstein, Nichols and Chaikoff,[96]

TABLE XI

Per Cent Oxidation of Cholesterol-26-C^{14}, Sodium Pyruvate-2-C^{14} and Sodium Octanoate-1-C^{14} by Mitochondria of Liver from Rats Fed Nicotinic Acid (0.75 per cent in Diet)*

Substrate	Experiment No.	Per Cent Oxidation of Substrates†			
		Males		Females	
		Nicotinic	Control	Nicotinic	Control
Cholesterol	1	7.1	1.9	34.8	22.5
	2	14.9	2.2	15.0	13.9
	3	17.7	4.4	6.5	2.9
	4	5.7	5.3		
	5	2.1	2.1		
Pyruvate	1	3.1	1.9		
	2	1.7	1.4		
Octanoate	1	13.9	24.3	21.5	25.1
	2	20.5	26.8	30.2	35.6

* From: KRITCHEVSKY, D., WHITEHOUSE, M. W. and STAPLE, E. *J. Lipid Res.*, 1:154, 1960.[92]
† All values are corrected for equivalent amounts of mitochondria (mg. N).

TABLE XII

Per Cent Oxidation of Cholesterol-26-C^{14} by Fortified Mitochondria of Rat's Liver*

Mitochondria from Rats	Supernatants from Rats	Per Cent Oxidation† Experiment No.		
		I	II	III
Fe-fed	Fe-fed	4.4	2.1	2.6
	Control	1.4	1.6	2.0
	None‡	1.0	0.1	0.5
Co-fed	Co-fed	3.3	3.6	2.1
	Control	2.2	2.5	3.5
	None‡	2.7	0.1	0.4
Ni-fed	Ni-fed	2.8	3.0	1.8
	Control	1.3	4.1	1.7
	None‡	1.1	Lost	0.7
Control	Control	1.3	3.9	0.9
	Fe-fed	3.6	3.9	1.6
	Co-fed	4.3	4.5	1.2
	Ni-fed	2.3	4.6	1.1
	None‡	0.2	0.2	0.4

NOTE: Incubation flask contains 1 ml. of mitochondrial preparation, 1 ml. of co-factors,[90] 5 ml. of boiled supernatant and 5 ml. of substrate solution in tris[hydroxymethyl]aminomethane HCl buffer, pH 8.5. All values corrected for equivalent amounts of mitochondria (mg. N).

* From: WHITEHOUSE, M. W., STAPLE, E. and KRITCHEVSKY, D. *Arch. Biochem.*, 87: 193, 1960.[94]

† Per cent oxidation computed as $BaC^{14}O_3$/cholesterol-26-C^{14}.

‡ 10% (w/v) sucrose used instead of boiled supernatant.

basing their work on the known ferric chloride precipitation of bile salts *in vitro*[97] prevented hypercholesterolemia in cholesterol-fed cockrels by feeding ferric chloride. Beher, Anthony and Baker[98] found, however, that cholic acid absorption in mice is not prevented by feeding ferric salts.

COMMENTS

The degradation of cholesterol to bile acids is thus seen to depend on the hepatic levels of bile salts. Cholesterol catabolism is accelerated under conditions in which bile acid turnover in the liver is increased. In some instances the serum cholesterol levels also reflect this situation. Thus, germ-free or hypothyroid animals, which exhibit longer half-lives of bile acid also have higher serum cholesterol levels, the reverse being true for hyperthyroid animals. Portman and Stare[99] have reviewed the effects of some other factors (phytosterols, vitamins, lipotropic agents, etc.) as they influence the serum cholesterol levels, presumably as a consequence of altering the relative rates of hepatic biosynthesis and catabolism.

Intestinal flora may exert marked effects on the serum cholesterol levels of animals fed cholesterol plus various carbohydrates.[100,101] Inasmuch as the intestinal flora are also implicated in the turnover of bile acids, their role in governing cholesterol homeostasis in the host should not be overlooked.

SUMMARY

The formation of cholesterol by the liver, regulated by a type of homeostatic control, is influenced by the hepatic level of free cholesterol or glycogen, or both, and by the availability of the proper enzymes. Here the factors affecting the hepatic biosynthesis of cholesterol are detailed, including the influence of dietary composition and fasting. The specific effects on cholesterol synthesis of feeding cholesterol, various steroids, saturated and unsaturated fats and thyroid hormone are reviewed. Also considered are the effects of radiation, glycolysis and various pharmacologic agents.

In the degradation of cholesterol, the chief metabolic end products are bile acids, although cholesterol may also be converted into sex hormones and adrenocortical hormones. The steps involved in the degradation of cholesterol to bile acids are reviewed, along with the various factors which increase or decrease cholesterol catabolism. Since degradation of cholesterol depends on the levels of bile salts in the liver, any factor which will increase the turnover of bile acids will also accelerate cholesterol catabolism and may in turn lower serum cholesterol.

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Vitamin B_6 and Lipid Metabolism in Monkeys

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THE USE of the monkey in nutrition experiments was stimulated by the studies of Waisman and co-workers[1] who found that rhesus monkeys (Macaca mulatta) could be maintained in good health on a purified diet providing that liver or liver extract was supplied in the ration. Day et al.[2–4] had earlier reported that rhesus monkeys, fed a modified Goldberger diet, succumbed to a leukopenia unless liver or yeast was added to the diet. McCall and co-workers[5] produced a vitamin B_6 deficiency in monkey (Macaca mulatta) characterized by microcytic anemia, mild leukopenia, polychromatophilia, presence of normoblasts, lack of growth, poor appetite and ataxia. Rinehart and Greenberg[6] observed arteriosclerotic lesions (intimal fibrosis) in vitamin B_6-deficient rhesus monkeys. Their findings were extended by Mushett and Emerson.[7] Greenberg and Rinehart[8] reported that the feeding of cholesterol to monkeys resulted in a greater elevation of serum cholesterol in pyridoxine-deficient animals than in the control animals although the latter consumed two to four times as much cholesterol as did the pyridoxine-deficient monkeys. Mann and co-workers,[9] although failing to produce hypercholesteremia in vitamin B_6-deficient Cebus and rhesus monkeys, succeeded in inducing hypercholesteremia and atherosclerosis in the Cebus monkey by the feeding of a diet high in cholesterol and low in the sulfur-containing amino acids. Cox and co-workers[10] produced hypercholesteremia and atherosclerosis in rhesus monkeys by the feeding of a diet high in cholesterol. Great variation was observed in the extent of cholesterol elevation. There was no consistent correlation between the intake of food or duration of feeding and the degree of elevation. Cholesterol levels of 300 to 600 mg. per 100 ml. were observed as contrasted with 120 to 150 mg per 100 ml. for a low fat regimen. The phospholipids also increased but not to the same extent as did the cholesterol.

Sclerosis of about 10 per cent of the small myocardial arteries and occasional thickening of the intima of the aorta were observed in the famous gorilla "Bushman" of Chicago's Lincoln Park Zoo.[11] Arteriosclerosis of the aorta and coronary and common iliac arteries of baboons reared in captivity for twenty years has been described by Lindsay and Chaikoff.[12] Atherosclerosis has been observed in the baboon in its natural habitat.[13]

The interrelationship between vitamin B_6 and fat metabolism is well documented. As early as 1938, Birch[14] reported that the addition of fatty acids to vitamin B_6-deficient diets tended to delay the onset of the acrodynia characteristic of the deficiency in rats as well as to mitigate its severity. Rats deprived of vitamin B_6 are unable to synthesize fat from protein[15] nor can they store normal amounts of body fat when fed a diet high in corn oil.[16] Quackenbush and co-workers[17] found that dermal lesions, which developed in rats fed a purified diet deficient in fat and vitamin B_6, could be cured by the administration of linoleic acid. Witten and Holman[18] have re-

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ported that pyridoxine is involved in the synthesis of highly unsaturated fatty acids from linoleate and linolenate. Pyridoxine plus linoleate relieved the acrodynia, stimulated growth, and promoted fat synthesis and arachidonate synthesis far more than did either supplement by itself. The combined administration of pyridoxine and linolenate stimulated growth, fat synthesis and hexaenoic acid synthesis to a greater extent than when they were given separately; however, dermal lesions were not affected.

Exclusion of fatty acids from the diet of young swine led to the production of gross lesions of the aorta.[19]

Greenberg and Moon[20] have recently studied the effects of combined deficiencies of the essential fatty acids and vitamin B_6 in the monkey. Monkeys deprived of essential fatty acids for two years were in good condition and had only minimal arteriosclerosis. However, monkeys deficient in both essential fatty acids and pyridoxine failed rapidly and required periodic supplements of pyridoxine. Arteriosclerosis was of the same order of severity as observed in vitamin B_6 deficiency. Dietary deficiency in the essential fatty acids did not cause significant change in serum cholesterol.

The study reported herein was designed to demonstrate the affect of graded levels of saturated fat (butter fat) and highly unsaturated fat (safflower oil) upon the clinical condition and plasma lipids of rhesus monkeys maintained for successive periods on graded levels of pyridoxine·HCl. Butter fat contains approximately 4 per cent of linoleic acid whereas safflower oil contains 78 per cent of linoleic acid.

METHODS

Twenty-six male rhesus (Macaca mulatta) monkeys with an average initial weight of 3.4 kg. were maintained on a diet of natural foodstuffs (Table I) for six months. At the end of this period the average weight was 4.1 kg. The condition of the monkeys and the biochemical and hematologic values for this period were considered to be within normal limits. The monkeys were then segregated into the dietary groups given in Table II. Each group consisted of four monkeys except for three animals which were assigned to the rations containing 10 per cent fat. Insofar as possible monkeys of like weights were placed in these groups. The diets were patterned after a purified ration employed at the Merck Institute for Therapeutic Research. A pilot study indicated that the rhesus monkey will consume a maximum of 20 per cent fat in the diet. The

TABLE I
Natural Food Diet*

Item	No.
Monkey pellets (Purina)	25
Whole wheat bread (slices)	1
Oranges	1/2
Apples	1/2
Bananas	1/2

* Average intake = 975 calories/day; (approximately 5 per cent fat).

TABLE II
Composition of Purified Diets (gm. per 100 gm.)

Diet	Labco Casein	Sucrose	Salts Wisconsin IV	Butter Fat	Safflower Oil
Fat-deficient	24	72	4	—	—
2% butter fat	24	70	4	2	—
10% butter fat	24	62	4	10	—
20% butter fat	24	52	4	20	—
2% safflower oil	24	70	4	—	2
10% safflower oil	24	62	4	—	10
20% safflower oil	24	52	4	—	20

Addendum to Diet (gm./100 gm.)

Inositol	0.04
p-Aminobenzoic Acid	0.04
Choline Cl	0.10
α-Tocopherol	0.015
Vitamin A & D (Pfizer's crystalettes) (500,000 units A and 50,000 units D/gm.)	0.003

Vitamin Supplements (Fed Daily)

Tablet

Thiamine·HCl	1 mg.
Riboflavin	1 mg.
Calcium pantothenate	3 mg.
Niacinamide	5 mg.
Biotin	20 μg.
Folic acid	500 μg.
Vitamin B_{12}	25 μg.

Plus sufficient powdered sucrose to make a 2 gm. tablet.

Fed on Sugar Cubes

Vitamin K (menadione)	1 mg.
Ascorbic acid	25 mg.

levels of pyridoxine fed daily and the duration of the periods were as follows:

Period	Mg.	Weeks
I	0.05	16
II	0.10	8
III	0.50	8
IV	1.00	8
V	2.00	8

The 0.05 mg. of pyridoxine was the lowest level employed by Rinehart and Greenberg.[21]

The monkeys were weighed semi-monthly and daily records were kept of their food consumption. The animals were trained by offering only a slight excess of diet: the food given to each monkey was based upon the average of that actually consumed by him during the previous two to three days. The cholesterol level was determined semi-monthly by the method of Sperry and Webb.[22] Plasma lipids were fractionated on silicic acid columns using a solvent modification of the method of Fillerup and Mead[23] (Table III).

The total number of determinations for all groups was as follows:

Period	No. of Monkeys	Cholesterol (in duplicate)	Lipid Fractions
Control (natural food diet)	26	78	52
I	26	156	78
II	26–22	48	48
III	22–12	30	30
IV	12–11	22	22
V	11	22	22

Routine hematologic studies were made by Dr. Drake Will of the Department of Pathology, Medical Center, University of California, Los Angeles.

OBSERVATIONS AND COMMENTS

The food intake decreased after the second week of daily feedings of 0.05 mg. of pyridoxine (period I), irrespective of the fat or its level: the average ranging from 110 gm. on the fat-deficient diet to 150 gm. on the 20 per cent safflower oil ration (Fig. 1). All animals gained in weight during the first few weeks, but only the monkeys receiving 20 per cent butter fat, and 10 per cent and 20 per cent safflower oil were able to maintain or slightly exceed their initial weights while on this low intake of pyridoxine (Fig. 2). Loss of hair and ataxia were noted in all groups. Increasing the level of pyridoxine to 0.10 mg. daily (period II) did not materially benefit the monkeys as evidenced by the absence of substantial change in food consumption and in growth response. In fact, the intake of food decreased in each group and all animals lost weight except those fed the diet deficient in fat and those which received 2 per cent butter fat. Increasing the intake of pyridoxine to 0.50 mg. daily (period III) resulted only in inconsistent improvement in the monkeys' general condition and in their intake of food. Alopecia and ataxia persisted throughout these three periods.

Four animals, two of which had convulsions, died during period II (Table IV). Two monkeys with convulsions survived. An additional ten monkeys, six of which had convulsions, died during period III in which 0.50 mg. of pyridoxine was given daily. Animals died in each of the dietary groups except in the group which received 10 per cent butter fat, which was probably fortuitous. Rinehart and Greenberg[6] did not observe convulsions in their monkeys; however, Mushett and Emerson[24] reported convulsive seizures in monkeys in which vitamin B_6 deficiency was induced by the administration of desoxypyridoxine (the animals were fed a diet composed of natural foodstuffs).

TABLE III

Separation of Plasma Lipids on Silicic Acid Column

Fractions	Solvent Mixtures	Ml.
I, sterol esters	3% ether in pentane	150
II, triglycerides	10% ether in pentane	200
III, sterols	20% ether in pentane	300
IV, monoglycerides, diglycerides and free fatty acids	100% ether	100
V, phospholipids	75% methyl alcohol in 25% ether	400

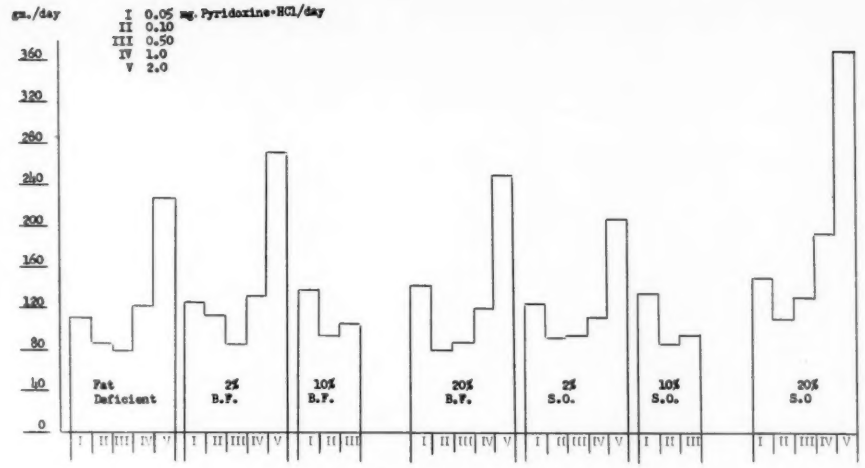

FIG. 1. Food intake (average).

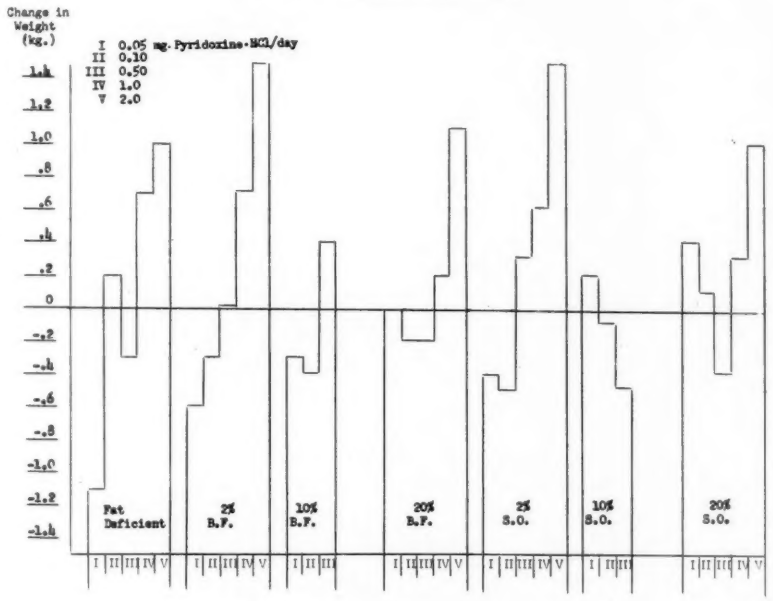

FIG. 2. Weight changes (average).

Because of the loss of monkeys, the groups were reconstituted as follows: groups which received levels of 10 per cent fats were discontinued; two animals receiving 10 per cent butter fat were placed in the 2 per cent butter fat group; and one animal receiving 10 per cent butter fat and one given 10 per cent safflower oil were transferred to the 20 per cent safflower oil group.

The food consumption increased in all groups when the intake of pyridoxine was raised to 1 mg. daily (period IV). The growth response was also augmented. The general condition of the animals improved; growth of hair and increased activity were noted. At the beginning of period IV, one animal died, although there was no evidence that convulsions had occurred. Possible loss of thiamine content of the vitamin

TABLE IV
Deaths and Convulsions

Diet	No. of Monkeys	I (16 wk.)	II (8 wk.)	III (8 wk.)	No. of Monkeys after Regrouping	IV (8 wk.)	V (8 wk.)
Fat-deficient	4			XC, XC	2		
2% butter fat	4		X, XC	X	3		
10% butter fat	3						
20% butter fat	4		C, C	X	2	X	
2% safflower oil	4		X	X, XC	1		
10% safflower oil	3			XC, XC			
20% safflower oil	4		XC	X, XC	3		
Total	26				11		

NOTE: I = 0.05 mg. Pyridoxine·HCl/day.
II = 0.10 mg. Pyridoxine·HCl/day.
III = 0.50 mg. Pyridoxine·HCl/day.
IV = 1.0 mg. Pyridoxine·HCl/day.
V = 2.0 mg. Pyridoxine·HCl/day.
X = Death without convulsions.
XC = Death with convulsions.
C = Convulsions with spontaneous recovery.

TABLE V
Effects of Thiamine Addition at 1 Mg. Pyridoxine Level
Growth Response

Diet	Change in Weight (kg.)		Food Consumption (gm. per day)	
	Vitamin Tablet*	+1 Mg. Thiamine†	Vitamin Tablet*	+1 Mg. Thiamine†
Fat-deficient	0	+0.3	107	97
2% butter fat	+0.1	+0.1	102	124
20% butter fat	+0.1	−0.1	125	106
2% safflower oil	+0.1	−0.4	67	100
20% safflower oil	+0.4	−0.3	168	167

Free and Total Cholesterol (mg. per 100 ml. plasma)

Diet	Vitamin Tablet‡		+1 Mg. Thiamine§	
	Free	Total	Free	Total
Fat-deficient	24.77	91.73	25.76	86.34
2% butter fat	27.49	112.90	35.21	134.80
20% butter fat	38.93	155.59	42.10	168.37
2% safflower oil	21.19	116.55	34.40	130.10
20% safflower oil	25.03	103.70	33.13	161.99

* Two weeks prior to thiamine addition.
† Two weeks after thiamine addition.
‡ Three weeks prior to thiamine addition.
§ One week after thiamine addition.

TABLE VI
Approximate Caloric Intake

Diet	Calories/Gm. Food	Mg. Pyridoxine·HCl per Day				
		(I) 0.05	(II) 0.10	(III) 0.50	(IV) 1.0	(V) 2.0
Fat-deficient	3.84	434	334	311	472	883
2% butter fat	3.94	500	372	370	441	823
10% butter fat	4.34	599	382	425	—	—
20% butter fat	4.84	726	528	639	944	1,805
2% safflower oil	3.94	500	449	339	516	1,076
10% safflower oil	4.34	599	399	464	—	—
20% safflower oil	4.84	707	387	426	586	1,215

TABLE VII
Pyridoxine·HCl Intake per kg. Body Weight (Average)

Diet	Mg. Pyridoxine·HCl per Day				
	(I) 0.05	(II) 0.10	(III) 0.50	(IV) 1.0	(V) 2.0
Fat-deficient	12	26	135	233	408
2% butter fat	13	27	139	245	388
10% butter fat	13	30	127	—	—
20% butter fat	13	25	132	267	449
2% safflower oil	12	26	147	282	460
10% safflower oil	12	25	127	—	—
20% safflower oil	12	24	141	282	494
Average	12	26	135	272	440

tablets was suspected when the container was found open which allowed exposure of some of the tablets to the moisture in the cold room. The values for thiamine content of these tablets (as determined by the thiochrome method) were inconsistent, probably due to difficulty in extraction. The daily feeding of 1 mg. of thiamine, in addition to the tablet, had no apparent affect upon the food intakes, the weights and the plasma cholesterol values of the monkeys (Table IV).

During period V all vitamins were fed on sugar cubes. Two mg. of pyridoxine were given during this period. A spectacular elevation in the consumption of food and a steady increase in growth were noted in all the monkeys except for those fed the fat-deficient diet. The animals maintained on this regimen showed only a modest growth response despite a substantial increase in the consumption of food. The greatest growth responses were observed in the groups fed 2 per cent butter fat and 2 per cent safflower oil.

The caloric intake (Table VI), with two exceptions, was the lowest for the monkeys receiving the fat-deficient ration. In general, the diets of the highest caloric content were consumed in the greatest quantity. This was particularly true for the monkeys fed 20 per cent butter fat; however, the average for this group was raised by one animal whose gains in weight were typical for the treatment although its intake of food approximately doubled that of its partners.

The growth responses observed with graded levels of pyridoxine would appear to indicate that the daily requirement of the growing rhesus monkey for this vitamin is approximately 1 to 2 mg. This quantity (which is average for all diets) represents an estimate of 0.26 to 0.44 mg. per kg. of body weight (Table

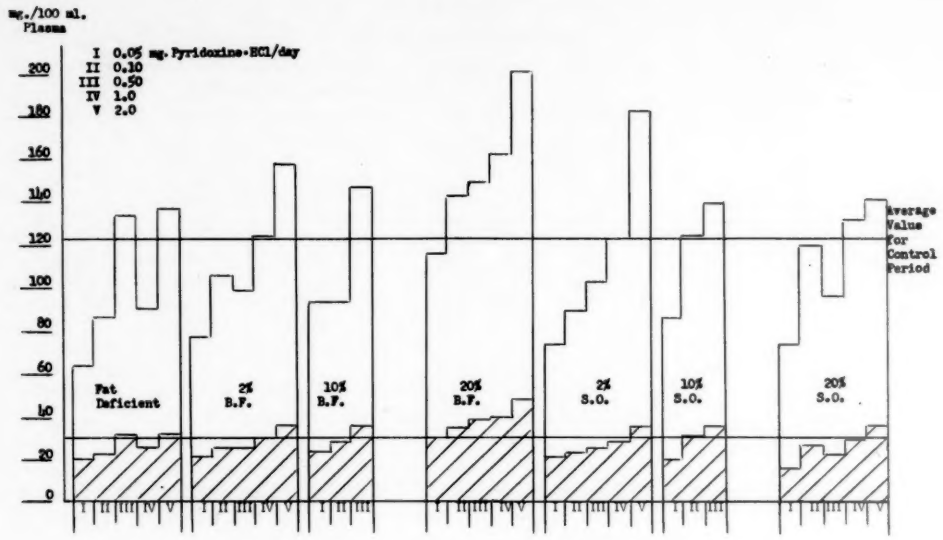

FIG. 3. Free and total plasma cholesterols (average).

VII), a level higher than the 0.062 mg. per kg. of body weight found by Rinehart and Greenberg.[21] These investigators, however, employed a diet containing 2 per cent corn oil which was lower in protein and in several of the vitamins than were the rations fed in the study reported herein. Mushett and Emerson[25] found that the daily administration of 1 mg. of pyridoxine did not invariably prevent fatty livers in rhesus monkeys.

The levels of total and free plasma cholesterol (Fig. 3), in general, increased with the intake of pyridoxine.

Butter fat contains approximately 300 mg. of cholesterol per 100 gm. The daily intakes of cholesterol ranged from 7.5 mg. on the regimen containing 2 per cent butter fat and 0.05 mg. pyridoxine, to 150 mg. on the diet containing 20 per cent butter fat and supplemented with 2 mg. pyridoxine daily. The fat-deficient and the safflower oil diets were devoid of cholesterol.

The values for total and free plasma cholesterol for the animal receiving 2 per cent safflower oil exceeded those for the control period only at the 2 mg. level of pyridoxine intake (period v)—an observation in agreement with that found with 2 per cent butter fat. The monkeys receiving 10 per cent safflower oil

TABLE VIII

Fractionation of Lipids

Sterol Esters (mg. per 100 ml. plasma)

Diet	Daily Intake of Pyridoxine* (mg.)				
	(I) 0.05	(II) 0.10	(III) 0.50	(IV) 1.0	(V) 2.0
Fat-deficient	102.8	121.8	109.4	131.0	143.3
2% butter fat	108.1	120.1	117.5	142.0	167.8
10% butter fat	138.5	140.4	163.3	—	—
20% butter fat	156.6	193.0	174.3	165.6	221.3
2% safflower oil	88.4	119.5	108.3	151.3	162.9
10% safflower oil	99.4	119.9	130.8	—	—
20% safflower oil	141.6	144.8	125.0	138.7	145.6

* Average value for stock diet—155.1.

TABLE IX

Fractionation of Lipids

Phospholipids (mg. per 100 ml. plasma)

Diet	Daily Intake of Pyridoxine* (mg.)				
	(I) 0.05	(II) 0.10	(III) 0.50	(IV) 1.0	(V) 2.0
Fat-deficient	150.0	230.6	237.8	328.8	275.6
2% butter fat	225.3	255.6	225.0	320.4	279.3
10% butter fat	250.1	316.7	292.8	—	—
20% butter fat	303.9	320.1	379.3	399.0	425.4
2% safflower oil	226.9	253.7	255.3	255.7	297.9
10% safflower oil	249.9	309.4	262.5	—	—
20% safflower oil	217.3	218.3	221.9	271.3	274.5

* Average value for stock diet—283.1.

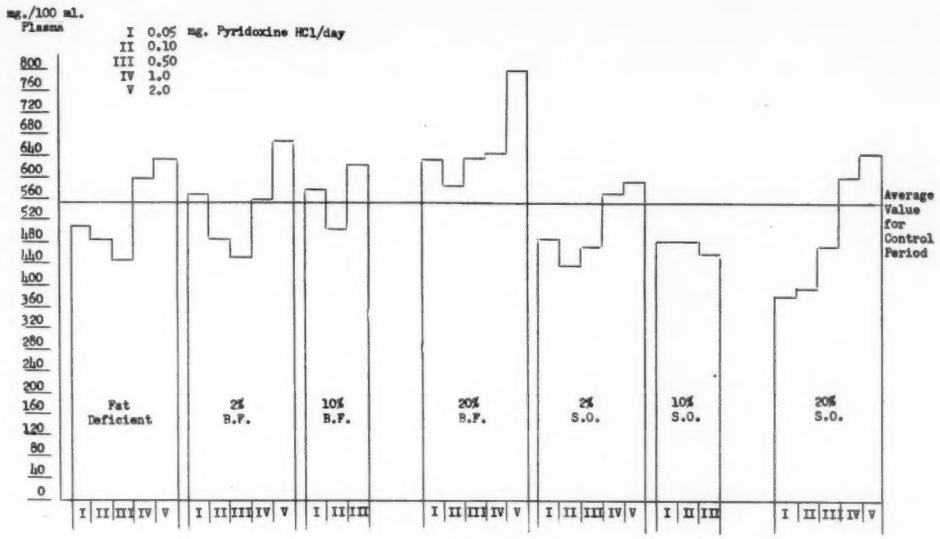

FIG. 4. Total plasma lipids (average).

also had essentially the same total and free plasma cholesterol levels as the corresponding groups fed 10 per cent butter fat. The total and free plasma cholesterol values for the groups fed 20 per cent safflower oil were appreciably lower than for the 20 per cent butter fat groups.

The patterns for sterol esters (fraction I, Table VIII) separated by silicic acid chromatography generally increased with the levels of pyridoxine and butter fat in the diets. Animals fed safflower oil showed a similar pattern; however, the maximal values were almost invariably lower than those for the corresponding groups receiving butter fat. The sterol esters increased in the fat-deficient group with the level of pyridoxine.

Triglycerides, free sterols and free fatty acids and mono and diglycerides (fractions II, III and IV, as eluted from the column) showed no consistent pattern.

The phospholipids (fraction V, Table IX) paralleled the plasma cholesterol values (Fig. 3) and, in general, increased with the level of pyridoxine within the specific fat group.

The total plasma lipids (Fig. 4) followed the patterns described for cholesterol and for phospholipids and generally were highest on intakes of 1 and 2 mg. of pyridoxine (periods IV and V). The highest value for plasma lipids was observed in the group receiving 2 mg. pyridoxine and 20 per cent butter fat. Total plasma lipids in the groups fed safflower oil were lower than those for the corresponding groups fed butter fat and were of the same order as observed for the fat-deficient groups receiving 1 and 2 mg. of pyridoxine. Total lipids for all groups receiving 1 and 2 mg. of pyridoxine (periods IV and V) were higher than those for the control period of stock diet feeding.

Complete hematologic studies were carried out at the end of each dietary regimen: the results were inconclusive. Hemoglobin and hematocrit values were higher during period V in which 2 mg. of pyridoxine were fed, than during the control period. Microcytosis and lymphopenia were not observed in any group. The level or type of fat in the diet appeared to be of little or no hematologic significance. Some animals in each of the groups, through 1 mg. of pyridoxine intake, showed a borderline and in a few cases a definite normocytic anemia. All values (red blood cell count, hemoglobin, hematocrit, white cell count and differential) were within the normal range for the groups receiving 2 mg. of pyridoxine.

SUMMARY

Graded levels of butter fat and of safflower oil (0, 2, 10 and 20 per cent) were fed to rhesus monkeys maintained for successive periods on graded levels of pyridoxine·HCl (0.05, 0.10, 0.50, 1 and 2 mg. daily). Effects of these regimens were observed in reference to clinical condition, food consumption, weight response, plasma lipids and hematology.

The intake of food decreased in all groups during the periods of feeding 0.05 and 0.10 mg. of pyridoxine. A slight increase was noted at the 0.50 mg. level in some groups and sustained increases were seen throughout the period of feeding 1 and 2 mg. In general, the intake of food increased with the caloric content of the diet.

Only the animals receiving 20 per cent butter fat and 10 and 20 per cent safflower oil maintained or slightly exceeded their initial weights during the period in which 0.05 mg. of pyridoxine was fed. Alopecia and ataxia developed in all groups. Increasing the intake of pyridoxine to 0.10 mg. daily for two months did not appreciably change the growth responses.

Inconsistent improvement was noted at the 0.50 mg. level of pyridoxine intake; however, the alopecia and ataxia persisted. Fourteen (of an initial twenty-six) monkeys died during the periods of feeding of 0.10 and 0.50 mg. of pyridoxine; eight of these had convulsive seizures. Two additional animals with convulsions recovered spontaneously. The groups on the 10 per cent level of the two fats were discontinued and the remaining animals served as replacements in other groups. The growth responses and the general condition of the monkeys including growth of hair and increase in activity improved during the period of feeding 1 mg. of pyridoxine. Further increases in weight occurred when the level of pyridoxine was raised to 2 mg. daily. Groups receiving the rations containing 2 per cent butter fat and 2 per cent safflower oil showed the greatest growth responses.

The levels of free and total plasma cholesterol, sterol esters and phospholipids increased with the intake of pyridoxine. These fractions, in general, were highest on the diets containing 20 per cent butter fat and lowest for the fat-deficient and the 20 per cent safflower oil diets. The total lipids followed a similar pattern. Total and free plasma cholesterol values for the monkeys given 20 per cent butter fat were greater than for the control period at all levels of pyridoxine intake except the lowest level. At the 1 mg. level of pyridoxine feeding the increment was 33 per cent over the control period and 65 per cent at the 2 mg. level of pyridoxine intake. Corresponding increases for the monkeys receiving safflower oil were 7 per cent and 15 per cent, respectively.

Mild to definite normocytic anemia was observed in some monkeys in each group other than at the 2 mg. level of pyridoxine intake.

The elevation of plasma lipids and their fractions appeared to be dependent upon the intake of pyridoxine and the level and type of fat in the diets.

ACKNOWLEDGMENT

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Vitamin B_6, Serum Lipids and Placental Arteriolar Lesions in Human Pregnancy

A Preliminary Report

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THE role of pyridoxine deficiency in the production of atherosclerosis in man is unknown, although animal experiments suggest a possible relationship.[1–5] Pyridoxine deficiency is not easily produced in normal human subjects under controlled conditions, but biochemical studies suggest that such deficiency frequently occurs during pregnancy unless supplementary pyridoxine is administered.[6–13] The human placenta has a marked tendency to develop sclerotic changes in its arterial vessels in a relatively short period of time during the course of "normal" pregnancy. Whether these changes were in any way related to the occurrence of pyridoxine deficiency during gestation had not been determined. Thus it appeared that study of the vessels of the placentas obtained from two groups of subjects, one receiving no supplementation with pyridoxine during pregnancy, and the other receiving doses of the vitamin adequate to maintain normal levels of xanthurenic acid excretion after administration of the tryptophan load test, might yield valuable information concerning the role of pyridoxine in the production of vascular changes. In addition, study of the lipid components of maternal and fetal blood and of the placental vessels would add to our limited knowledge of the role of pyridoxine in human lipid metabolism.

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EXPERIMENTAL PROCEDURE

Thirty-three patients are included in this preliminary report. Additional patients are currently under study. All subjects were selected from the Tulane Obstetrical Unit of the Charity Hospital of Louisiana at the time of their initial visit to the prenatal clinic. All the patients are Negro. Only those were selected who had no evident disease or complication of pregnancy and who were not more than three and a half months pregnant. Since the experimental plan required that each patient be admitted to the hospital once monthly for a twenty-four–hour period during the course of gestation, it was necessary to select only those who stated that they were willing and able to cooperate in this manner. Seventeen patients were lost from the study because of their failure to cooperate, and an additional seven patients were eliminated because of failure to obtain the placenta at the time of delivery. One patient was lost to the study because of a spontaneous early abortion at home.

Patients were assigned by a standard double-blind procedure to one of the two study groups. One of the groups received a daily supplement

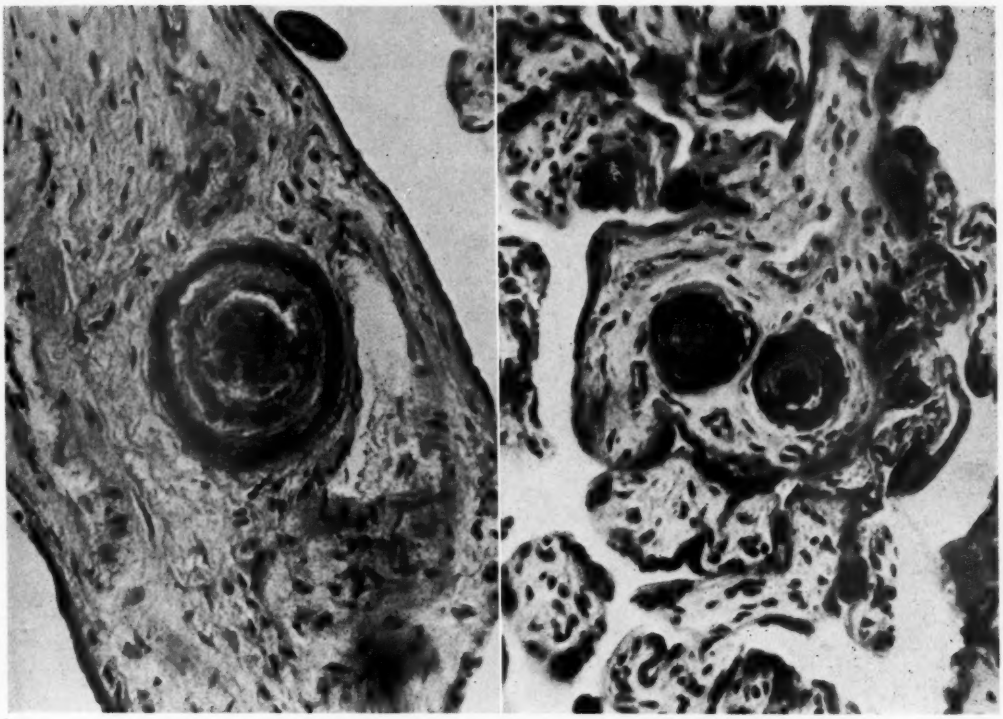

FIG. 1. Calcification of arterioles of villus. Original magnification × 360.

of 25 mg. of pyridoxine hydrochloride, the other a placebo of identical appearance. Routine prenatal care was given each patient by one of us in accordance with current policies and practices of the Tulane Obstetrical Unit in the Charity Hospital prenatal clinics. In addition, at the time of each patient's visit a dietary history was taken by a trained dietitian and the patient's nutritional status evaluated by a physician of the Tulane Nutrition and Metabolism Unit.

As soon as possible following admission to the study, and at monthly intervals thereafter until the time of delivery, and one and two months postpartum the patient was admitted to our metabolic ward. At that time the xanthurenic acid excretion test was performed, and blood samples were obtained for the determination of serum lipids, hematocrit and blood urea nitrogen.

At the time of delivery additional blood samples were obtained from the patient and a sample of umbilical cord blood was obtained for lipid studies. The placentas were refrigerated temporarily until they could be frozen to await subsequent examination.

Xanthurenic acid excretion in twenty-four–hour urine samples, following the administration of a test dose of 10 gm. DL-tryptophan, was measured by the technic of Wachstein and Gudaitis.[14] Free and total cholesterol of the serum was measured in ethanol-diethyl ether extracts by the method of Sobel and Mayer,[15] lipid phosphorus by the method of Horecker and associates,[16] and esterified fatty acids by the hydroxamic acid method of Hack.[17] Blood urea nitrogen was determined by a modified Karr[18] technic. Wintrobe hematocrit determinations were performed on oxalated samples of venous blood.

The placental tissue was prepared for microscopic examination by a special procedure. The larger arteries on the fetal surface of the placenta were dissected away from the placenta,

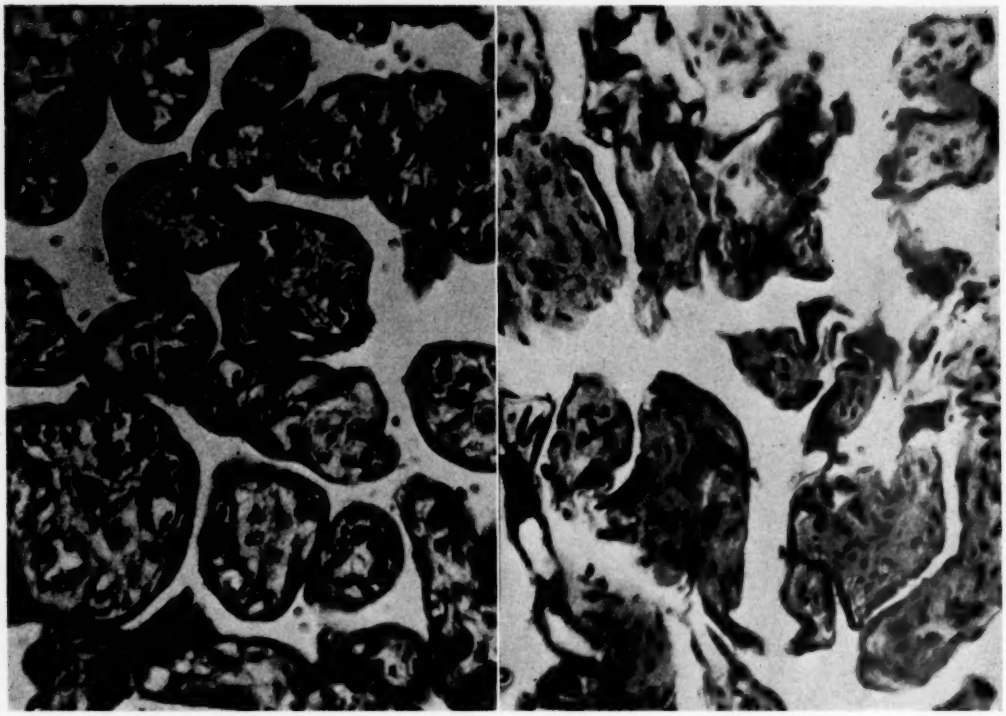

FIG. 2. "Young" and "old" placental villi. Original magnification × 360. *Left*, young villi with patent arterioles, syncytium and large vesicular connective tissue nuclei. *Right*, hyalinized, fibrotic villi. Note clumping of syncytium and small pyknotic nuclei.

then tied together in bundles of three or four, and placed in formalin. For the smaller vessels of the placenta the cotyledons were separated by blunt dissection from surrounding cotyledons and from the basal portion of the placenta, until the cotyledonary stalk was exposed. The soft tissues were stripped away from the cotyledon by means of the fingers, leaving behind the fibrous tissue which contained the majority of the blood vessels. All the available cotyledons from the placenta were prepared thus, and placed together with the basal ends aligned at the same end of the group. This group of cotyledonary fibrous tissues was tied together with catgut sutures, and then placed in formalin, with the basal end identified. The tissue blocks were made from first, the group of larger arteries, second, a block across the cotyledonary tissues at the basal end, and third, a block at the distal end of the cotyledonary tissue. This procedure allowed larger numbers of smaller vessels to be placed upon a single slide, reducing greatly the number of blocks that had to be prepared. It did not interfere with the structure of the vessels, and quite satisfactory cuts were made. The sections were stained with hematoxylin and eosin, Van Gieson's elastic tissue stain, toluidine blue stain, colloidal iron and aldehyde fuchsin stain.

The placental vessels were studied by two observers independently. The slides were labeled by number, and only after the evaluation was completed were the placentas identified. In most instances, the grading of vascular change by the two observers was in agreement, and in no case was there marked difference of opinion.

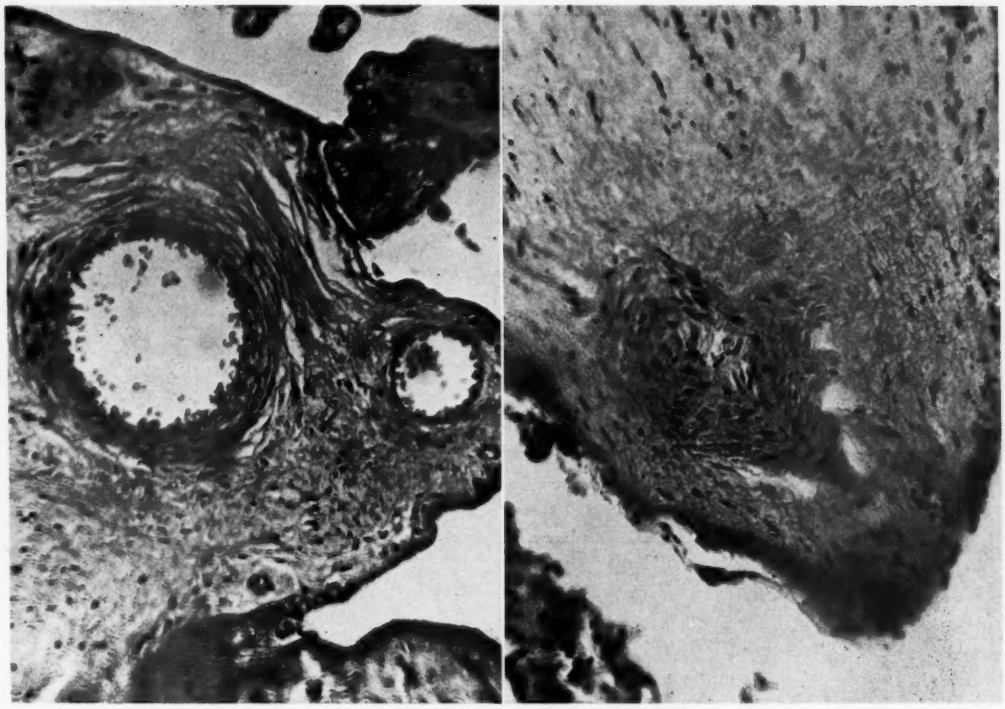

FIG. 3. Septal arterioles of placenta. Original magnification × 260. *Left*, patent arteriole in "young" placental septum. *Right*, obliterated lumen of "old" septal arteriole.

Atheromatous plaques were not found in the vessels of the placenta. The narrowing and the occlusion of the lumen of the arterioles was the result of intimal proliferation and occasional thrombus formation. Another difference in the lesions in the placenta from atherosclerosis as seen in larger vessels is that degeneration of elastic tissue was not discernible because of the small amount of such tissue in these vessels. Calcification of fibrous tissue of the septums and villi was commonly seen, but in only two cases involving three small vessels was calcification of the vessel walls observed (Fig. 1).

Because of these differences from classic atherosclerosis, the condition of the arterioles of the placenta was evaluated by the degree of proliferation and the condition of the intima, by medial degeneration or thickening and by the patency of the lumen. However, the changes observed in the arterioles of the placenta are similar to those seen in arteriolarsclerosis rather than arteriosclerosis of the adult human being.

In order to estimate "age" or degree of "ripeness" of the placenta, the periarteriolar connective tissue and the syncytium of the villi were studied. In Figure 2 "young" villi with abundant and patent vessels, quite good syncytium and large vesicular nuclei, are contrasted with the hyalinized, fibrotic villi of the more mature placenta in which little or no blood supply is apparent. The syncytium has become clumped on one side of the villus, and the nuclei of the connective tissue cells have become pyknotic. Similar changes in the larger arterioles of the placental septums are seen in Figure 3.

With these changes being considered, the placentas were divided into three groups: (1)

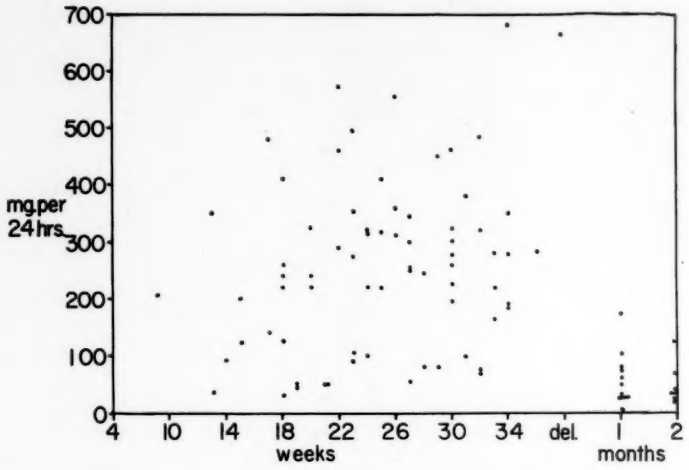

FIG. 4. Control group. Xanthurenic acid values after initial determination.

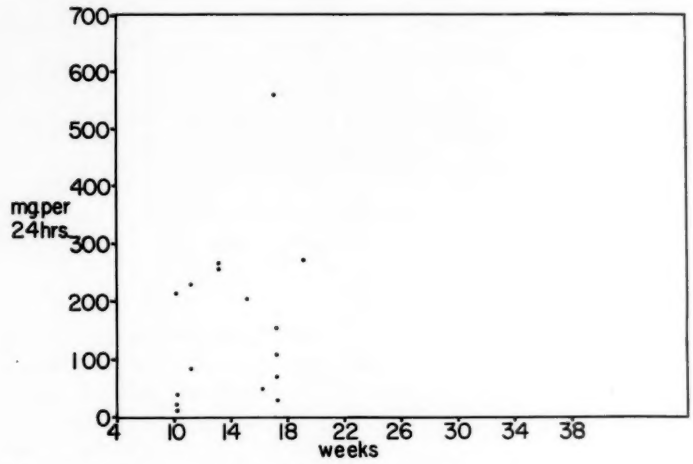

FIG. 5. Group receiving supplemental pyridoxine. Initial xanthurenic acid values.

normal with only slight change; (2) moderately advanced change; and (3) markedly advanced change.

RESULTS

Patients of both groups were subjected to careful physical examination for evidence of nutritional deficiency at the time of each visit. No signs were observed which could be attributed to specific deficiency disease. Mild degrees of nasolabial seborrhea were noted in six members of each group. No relationship could be demonstrated between the occurrence of these lesions and the level of xanthurenic acid excretion in the urine following the tryptophan load test. Seborrhea usually persisted throughout the period of gestation, and was uneffected by the administration of pyridoxine. No significant glossitis, cheilosis, angular stomatitis or evidence of neuropathy was encountered in either group.

There were no prenatal complications of serious nature, or complications of delivery in the group included in this report. All the

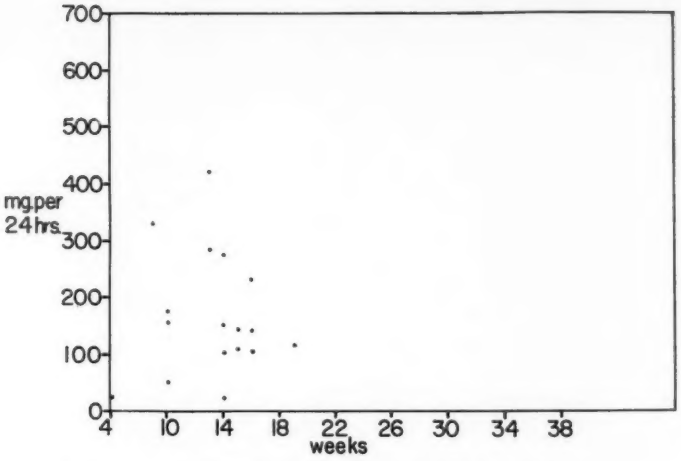

FIG. 6. Control group. Initial xanthurenic acid values.

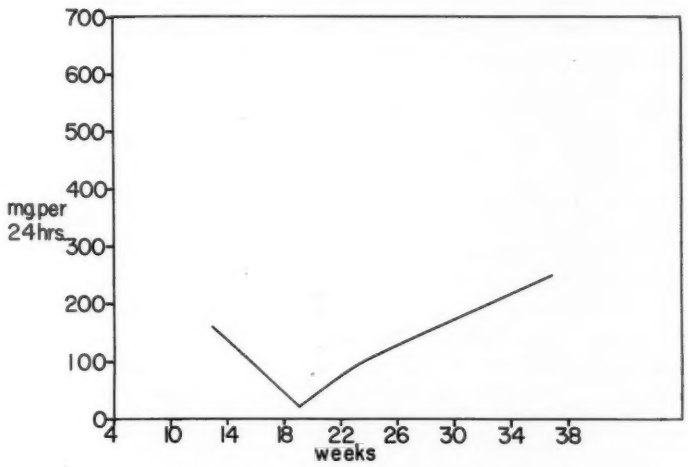

FIG. 7. Patient 104. Xanthurenic acid values.

babies were normal except one, who died of acute leukemia at the age of eight weeks.

The mean xanthurenic acid output in the control group was high throughout pregnancy. It rose from a value of 183 mg. in the third month to a value of 89 mg. in the ninth month. In two control patients xanthurenic acid values during pregnancy remained essentially normal. In six others of the control group all xanthurenic acid values were abnormally high. The remainder of the patients in the control group had random xanthurenic acid values that were below 50 mg. In five determinations the values were over 500 mg. (Fig. 4).

The mean total cholesterol value for the control group rose from 155 mg. per 100 ml. in the third month to slightly over 200 mg. per 100 ml. during the seventh, eighth and ninth months. The over-all tendency was for a progressive rise to the seventh month and then a leveling off until term. One month postpartum the average cholesterol value in this group was 193.7 mg. per 100 ml., and three months' post-

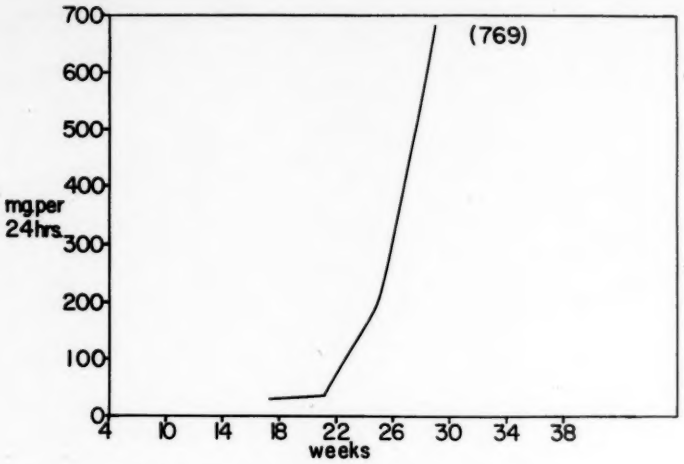

FIG. 8. Patient 108. Xanthurenic acid values.

partum the average value was 150 mg. per 100 ml. This generally agrees with the work of Smith et al.[19,20]

Free cholesterol in the control group during the third month was 40.2 mg. per 100 ml. This rose to between 53 and 54 mg. per 100 ml. in the seventh, eighth and ninth months.

Phospholid levels, as measured by lipid phosphorus, increased up to the seventh month and remained at those levels through the eighth and ninth months.

In the control group two placentas had normal-appearing arterioles, seven had moderate changes and nine had marked changes.

The group receiving supplements varied in several respects from the control group. The initial mean xanthurenic acid value, excluding three patients who were inadvertently started on pyridoxine before the initial test, was 167 mg., which is identical with the control group (Figs. 5 and 6). In the group receiving supplements after the initial observation, all the xanthurenic acid values were below 50 mg., with the exception of two patients (Figs. 7 and 8). It is possible that these patients failed to take the vitamin supplement after a period of time.

Total and free cholesterol values tended to be somewhat lower than in the control group (Fig. 9). The difference is not significant because of the small numbers in each group; however, it suggests that there may be a difference. Lipid phosphorus values were also somewhat lower (Fig. 10).

Table 1 shows the total and free cholesterol values for the two groups at delivery. The fetal values at that time are shown also. The differences between the two groups are not significant; however this illustrates the gra-

TABLE I

Cholesterol Gradient Across the Placental Membrane (mg. per 100 ml.)

Group	Maternal		Fetal	
	Total	Free	Total	Free
Control	218	59.2	42.2	13.6
Receiving pyridoxine	192	52.2	36.7	11.0

TABLE II

Group	Last Maternal Weight Before Delivery (lb.)	Maternal Age (yr.)	Placental Weight (gm.)	Fetal Weight (gm.)
Control	140.4	24.3	514	3,130
Receiving pyridoxine	146.6	19.8	428	2,893

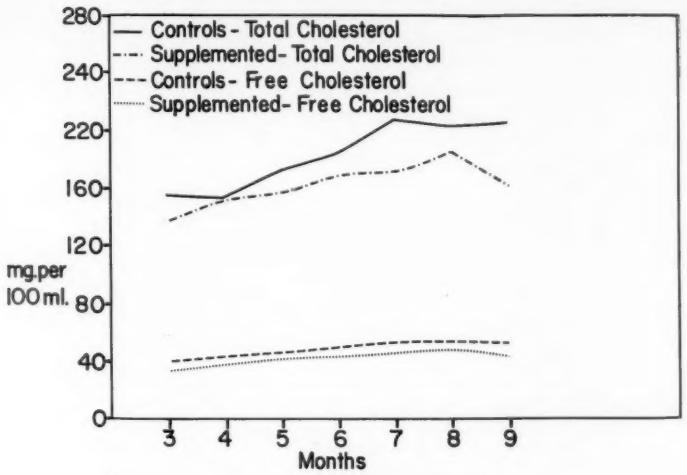

FIG. 9. Mean cholesterol values.

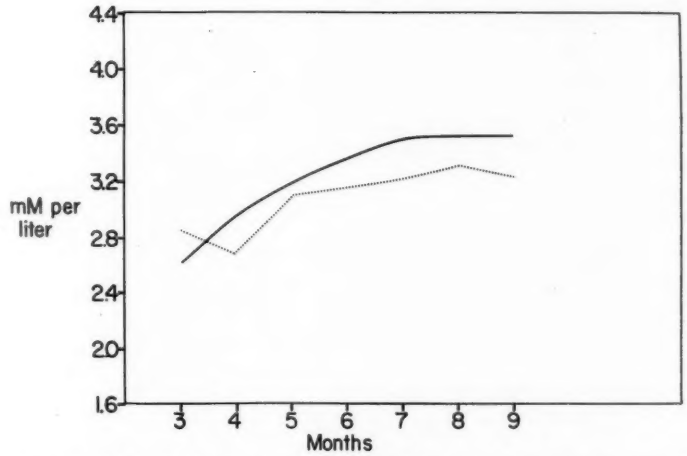

FIG. 10. Mean lipid phosphorus. Solid rule represents values for control group, broken line for group receiving supplemental pyridoxine.

dient for free and total cholesterol across the placental barrier.

The average weight gain in the control group was 18 pounds, and in the group receiving supplements 15 pounds. This is not total weight gain, since in a number of patients the weight before pregnancy was not known, and the weight at the time of the initial visit was used.

The mean last weight before delivery in the control group was 140.4 pounds, and in the group receiving supplements 146.6 pounds (Table II). However the mean placental weight for the control group was 514 gm. and for the group receiving supplements 428 gm. The mean fetal weight for the control group

TABLE III
Placental Arterioles

Group	Normal	Moderate Changes	Severe Changes
Control	2	6	9
Receiving pyridoxine	7	2	7

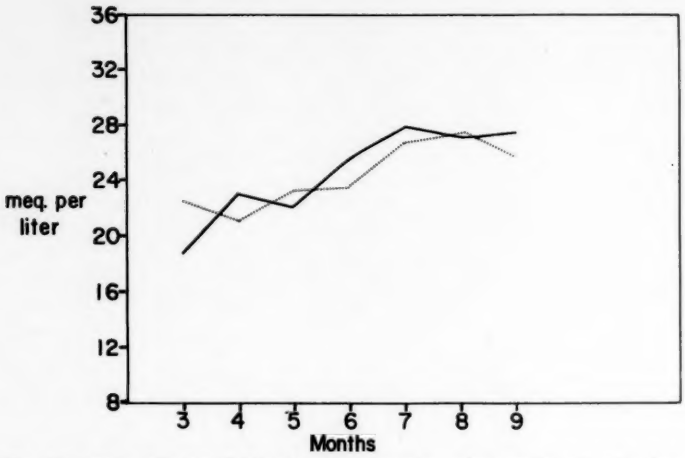

FIG. 11. Ester bonds. Solid rule represents values for control group, broken line for group receiving supplemental pyridoxine.

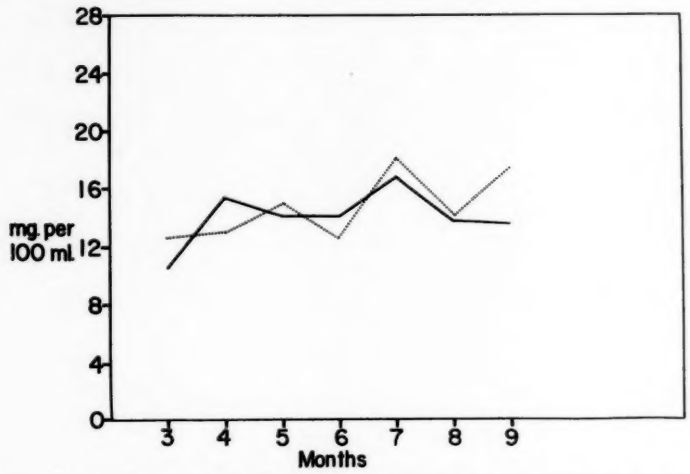

FIG. 12. Blood urea nitrogen. Solid rule represents values for control group, broken line for group receiving supplemental pyridoxine.

was 3,130 gm. and for the group receiving supplements 2,893 gm. These differences in placental and fetal weight are probably significant ($P = 0.02$ in each case). The evaluation of the placental vessels of these two groups is shown in Table III. With this distribution, $P = 0.04$ by the Chi Square test, which with this small group may not be significant.

Total ester bond values increased during pregnancy in both groups with the supplemented group having lower mean values. This is shown in Figure 11. The blood urea nitrogen values were not different in the two groups (Fig. 12).

A few short term studies were undertaken to evaluate the results of pyridoxine supplementation in pregnant women who showed an abnormally high excretion of xanthurenic acid. Four patients who were in the midtrimester of pregnancy were used. These patients were selected at random from the clinics, and are not part of the principal study. They had a variable initial control xanthurenic acid output (Table IV). After one

TABLE IV
Xanthurenic Acid Values
(mg. per 24 hr.)

Patient	Initial Value	After 25 mg. Pyridoxine Daily for 7 Days	Pyridoxine Discontinued for		
			1 Week	2 Weeks	3 Weeks
M. M.	336	36	119	..	440
O. B.	230	32	205	..	..
O. T.	228	9	14	131	..
E. S.	95	24	25	36	..

week's therapy with 25 mg. of pyridoxine a day, the xanthurenic acid outputs reverted to normal levels in all patients. After remaining off pyridoxine for one week two patients again had abnormal values. After two weeks of no pyridoxine, one patient reverted to an abnormal xanthurenic acid output, and a third patient who had an initial output of 95 mg. xanthurenic acid after three weeks without pyridoxine was still at a normal level. The initial level of xanthurenic acid excretion seemed to relate to the length of time necessary for reversion to abnormal values.

COMMENTS

This preliminary report is on a small group of patients so that the statistical evaluation is difficult. However, there is a suggestion that dietary pyridoxine supplements given to pregnant women resulted in changes in maternal serum lipids, fetal weights, placental weights and the condition of blood vessels in the placenta. In the group receiving supplemental pyridoxine, maternal serum lipid, fetal weight and placental weight were lower and a larger proportion of the placental arterioles were normal in appearance.

The differences in placental vessels suggest that pyridoxine deficiency may play a role in the development of arteriolar lesions in the placenta. Although the number of patients in this preliminary study is small, we believe that the study should be continued and sufficient numbers of patients obtained to give a better evaluation of any differences.

SUMMARY

A preliminary report is given on a study of the effects of sufficiency or deficiency of vitamin B_6 on placental vessels and serum lipids in human pregnancy. The women given a placebo had a high xanthurenic acid output throughout pregnancy and had more placental arteriolar changes than the group receiving supplements of pyridoxine. The two groups are too small for the vascular differences to be statistically significant. However, there was a significant difference in fetal and placental weight, with the group not receiving supplements having heavier babies and placentas. The serum lipid values were not different in the two groups, and both groups showed a high gradient across the placenta for cholesterol.

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Relation of Vitamin E to Lipid Metabolism

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A REQUIREMENT for vitamin E by various species of animals, excluding man, has long been recognized and the various ill effects observed as a result of a deficiency of vitamin E have been well documented. Our interest in the role that vitamin E exerts in some phases of lipid metabolism has arisen from three areas of our research: (1) the protection afforded polyunsaturated fatty acids in the body by the ingestion of vitamin E; (2) the production of experimental muscular dystrophy in certain species of animals when fed a vitamin E-deficient diet; and (3) the alleviation by vitamin E of interferences with reproductive performance in rats fed certain heat treated oils as the sole source of fat in the diet.

Reports concerning the relations between vitamin E and various phases of cholesterol metabolism are relatively few and are controversial in nature. The action of vitamin E in the maintenance of normal cholesterol levels in skeletal muscle is well substantiated. An elevation of the cholesterol levels in skeletal muscle has been observed in vitamin E-deficient rabbits, guinea pigs, rats, calves and chicks.[1-8] It has also been reported that vitamin E deficiency in rabbits and guinea pigs results in elevated plasma cholesterol levels,[4,7-9] although Gray and Loh[10] have reported an increased plasma cholesterol value in healthy subjects after tocopherol administration. Cholesterol levels in the liver are supposedly unaffected in vitamin E deficiency.[4,7] In experiments in which cholesterol was fed to both chicks and rabbits,[4,11] the marked increase of cholesterol in the liver which resulted was independent of the tocopherol content of the diet.

The activity which α-tocopherol exerts on tissue and plasma cholesterol levels may be mediated through its antioxidant effect on the protection of the polyunsaturated fatty acids, which are now known to be important in the regulation of some phases of cholesterol metabolism.[12] Several years ago in a study of the effect of a saturated animal fat, i.e., lard and an unsaturated vegetable fat, i.e., cottonseed oil on cholesterol levels of tissues of the rat, it was concluded that the type of dietary fat fed was an important factor, not only for its fatty acid composition but also with respect to the tocopherol content of the fat.[13] In feeding studies with rats, no significant differences were observed in plasma cholesterol levels until after twenty-four weeks on the diets when the plasma cholesterol levels in the rats fed cottonseed oil were markedly lower when compared with those fed lard. The differences in cholesterol levels in the liver were apparent as early as twelve weeks after the initiation of the two dietary regimens (Table I).

These changes were not due to dietary deficiency of essential fatty acids for two reasons: (1) An analysis of the fatty acid composition of the fats used in the feedings (Table II) revealed that the presence of 12 per cent dienoic (linoleic) acid in the lard (which corresponds to approximately 180 mg. of linoleic acid per 10 gm. of diet) should certainly have been adequate even in the presence of saturated fatty acids which increase the requirement for essential fatty acids.[14] (2) In the typical picture of the rat with an essential fatty acid

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TABLE I

Effect of Feeding Lard or Cottonseed Oil at a Level of 15 Per Cent in the Diet on Plasma and Liver Cholesterol Levels in Male Rats

Weeks on Diet	Lard		Cottonseed Oil	
	Plasma Cholesterol Level (mg. %)	Liver Cholesterol Level (mg./gm.)	Plasma Cholesterol Level (mg. %)	Liver Cholesterol Level (mg./gm.)
6	80.5	2.77	84.1	2.76
12	75.4	3.25	79.6	2.66
24	77.9	3.18	61.9	2.48

TABLE II

Fatty Acid Composition of Lard and Cottonseed Oil*

Composition	Lard	Cottonseed Oil
Iodine value	68	107
Saturated fatty acids	39	31
Monoenoic fatty acids	48	20
Dienoic fatty acids	12	49
Polyenoic fatty acids	1	..

* Per cent.

deficiency, the increase in liver cholesterol is accompanied by a decrease in plasma cholesterol and this was not the case in the animals fed lard. The lard used in this experiment was stripped of vitamin E but was protected by the addition of certain antioxidants (0.01 per cent propyl gallate and 0.02 per cent butylated hydroxy anisole) with 0.005 per cent citric acid in propylene glycol added as preservative.

However, in a determination of possible oxidation of the fats in the diets (by the thiobarbituric acid test), the amount of oxidized material present in the diet containing lard was approximately ten times that present in the cottonseed oil diet despite the fact that antioxidants were present in the lard, and that the lard contained much less potentially oxidizable material than did the cottonseed oil. On this basis it is possible that less dienoic acid than was originally present in the lard was ingested. However, tests of liver function performed on these animals did not reveal any impairment or damage as a result of the ingestion of oxidized fat.

TABLE III

Percentage Distribution of Fatty Acids of Cholesterol Esters and Phospholipids of Pooled Liver Extracts of Animals Fed a Lard Diet with and without Vitamin E

Composition	Cholesterol Esters		Phospholipids	
	Lard	Lard with Vitamin E	Lard	Lard with Vitamin E
Iodine value	78	79	96	147
Saturated fatty acids	30	27	68	49
Monoenoic fatty acids	59	64	..	11
Dienoic fatty acids	9	5	12	7
Trienoic fatty acids	..	1	4	5
Polyenoic fatty acids	2	3	16	28

The addition of vitamin E to the diet of the animals fed lard caused a decrease in cholesterol levels in the liver without affecting the plasma cholesterol levels. Analysis of the fatty acid composition of the cholesterol esters and phospholipids in the liver lipids of animals in these groups revealed that the presence of large amounts of tocopherol resulted in higher unsaturation of the fatty acids in the phospholipid fraction, although the fatty acid composition of the cholesterol esters is essentially unchanged (Table III). The action of vitamin E in this case would seem to be that of an *in vivo* antioxidant. Similar observations on changes in fatty acid composition resulting from vitamin E deficiency have been reported by Hove and Seibold,[15] who observed that lipids of the muscle and liver of vitamin E-deficient hogs had lower concentrations of dienoic, tetraenoic and pentaenoic acids than did those of vitamin E-supplemented control animals.

It is well known that a deficiency of vitamin E will produce an experimental muscular dystrophy in various species of animals. Attempts to determine whether or not this activity is linked to the antioxidant properties of this vitamin have been made by many investigators using a series of antioxidants; here again the literature is confusing and controversial. It is possible to explain the contradictory findings by variation in such factors as type of antioxidant used, size of dose, level and type of fat in the diet, and

TABLE IV

Comparative Effect of α-Tocopherol Acetate, DPPD and Other Antioxidants on the Incidence and Time of Onset of Muscular Dystrophy in Guinea Pigs Fed a Vitamin E-Deficient Ration*

Supplement	Incidence of Muscular Dystrophy (%)	Average Time of Onset of Muscular Dystrophy (days)
α-Tocopherol acetate	0	..
None	100	139
DPPD	62.5	235
Santoquin	87.5	182
DBH	100	231
BHT	75	240

* Eight animals per group.

most important, the duration of the experiment. Results of an investigation in which we compared the activity of several antioxidants with that of tocopherol in feeding studies with guinea pigs have been published.[8,16]

MATERIAL AND METHODS

Groups of guinea pigs which had been placed on a vitamin E free ration containing 30 per cent protein and 5 per cent stripped lard had the following antioxidants incorporated into their diet: (1) 0.025 per cent N,N′ diphenyl *p*-phenylenediamine (DPPD); (2) 0.025 per cent 6-ethoxy-2,2,4-trimethyl - 1,2 - dihydroquinoline (Santoquin®); (3) 0.025 per cent 2,5-di-tert-butyl hydroquinone (DBH); and (4) 0.025 per cent butylated hydroxytoluene (BHT). Two other groups of guinea pigs were included. One group was fed a diet supplemented with α-tocopherol acetate and the other group was given an unsupplemented diet. The results are shown in Table IV.

RESULTS

For the first twelve weeks of the feeding period there were no significant differences in weight or gross appearance among the various groups of guinea pigs. All animals survived and were indistinguishable from the guinea pigs on the control, tocopherol-supplemented ration. The average body weight at this time is shown in Table V.

TABLE V

Average Body Weight of Guinea Pigs During the First Twelve Weeks of Feeding

Supplement	Weight (gm.)
None	506
α-Tocopherol acetate	537
DPPD	534
Santoquin	545
DBH	510
BHT	515

During the thirteenth week, clinical symptoms of muscular dystrophy developed in three of the animals on the basal unsupplemented vitamin E-free ration. The animals dragged their hind limbs and had difficulty in turning over when they were placed on their backs. In no other groups were there dystrophic animals. At this time, the three dystrophic animals, one non-dystrophic animal from this group and four animals from each of the other groups were sacrificed and autopsies were performed. Blood and tissues were extracted for cholesterol and lipid analyses and sections of the gastrocnemius muscle were prepared for histologic analysis. The dystrophic animals exhibited the typical Zenker degeneration observed by Pappenheimer[17] and others as characteristic of the dystrophic voluntary muscles of vitamin E-depleted animals. The musculature of the other animals appeared normal in all respects. In contrast to results obtained on the basal vitamin E-free ration, clinical symptoms of muscular dystrophy did not develop in animals fed diets containing DPPD, Santoquin, DBH or BHT until after the twenty-fifth week of feeding, whereas muscular dystrophy did not occur in any of the animals to which α-tocopherol acetate was administered.

It may therefore be said that DPPD, Santoquin, DBH and BHT delayed but did not prevent occurrence of muscular dystrophy in guinea pigs; however, α-tocopherol acetate gave full protection to the animals in this respect. Since the basal diet employed in these studies was free of vitamin E and contained no cod liver oil or other highly unsaturated oils, it would appear that the delay in onset of mus-

TABLE VI

Effect of α-Tocopherol and Other Antioxidants on Plasma Cholesterol Levels of Guinea Pigs

Supplement	Plasma Cholesterol (mg. %)	
	Free	Total
Non-dystrophic Animals		
None	34.2	75.1
α-Tocopherol acetate	17.6	48.5
DPPD	24.9	91.3
Santoquin	24.1	77.0
DBH	33.6	91.1
BHT	25.7	68.8
Dystrophic Animals		
None	57.3	119.0

cular dystrophy in animals on diets containing DPPD, Santoquin, DBH and BHT was due not to the sparing effect of these antioxidants on residual quantities of vitamin E in the diet (which is readily destroyed in the presence of unsaturated fats, particularly fish oils) but rather to the sparing effect of these antioxidants on vitamin E in the tissues. As long as tissue stores of vitamin E were adequate, muscular dystrophy did not develop. When tissue stores were depleted, the antioxidants were ineffective in preventing muscular dystrophy in contrast to the continued effectiveness of α-tocopherol.

The results of the lipid analyses are reported in Tables VI and VII. There is a marked increase in both free and total cholesterol levels in plasma of dystrophic animals fed the unsupplemented vitamin E-deficient diet as well as in those fed the vitamin E-free ration to which the antioxidants other than tocopherol have been added (Table VI). This is in agreement with our previous experiments with vitamin E-deficient guinea pigs and rabbits[7] and with those of Morgulis and Spencer[1] in vitamin E-deficient rabbits. In the group with the unsupplemented vitamin E-deficient diet in which animals were sacrificed before the onset of the symptoms of muscular dystrophy, in what might be considered an early stage of

TABLE VII

Effect of α-Tocopherol and Other Antioxidants on Cholesterol and Total Lipid Levels in the Muscle of Guinea Pigs

Supplement	Cholesterol (mg./gm.)		Total Lipid (gm./100 gm.)	
	Non-dystrophic	Dystrophic	Non-dystrophic	Dystrophic
None	1.16	1.44	4.11	5.10
α-Tocopherol acetate	0.72	..	2.13	..
DPPD	1.13	1.01	3.66	4.05
Santoquin	0.98	0.90	2.69	2.68
DBH	1.17	1.72	2.89	2.98
BHT	1.05	1.40	3.84	3.13

the disease, there is also evidence of an increased concentration of plasma cholesterol. Elevations in plasma cholesterol levels are also observed in the groups of animals fed the vitamin E-free ration supplemented with antioxidants other than α-tocopherol, i.e., DPPD, Santoquin, DBH and BHT.

The elevations of cholesterol in skeletal muscle observed in dystrophic animals fed the vitamin E-free ration also confirm the earlier findings.[1-3,5-7] However, it can be seen in Table VII that these changes also precede the onset of muscular dystrophy since in the non-dystrophic animals either receiving diets with no supplement or supplemented with non-tocopherol antioxidants, an increase in skeletal muscle cholesterol is observed.

The total lipid content of skeletal muscle of animals on vitamin E-free unsupplemented diets is markedly higher than that of animals fed α-tocopherol both before and after the onset of paralysis. The lack of tocopherol activity of the other antioxidants is also evident here although not to the same extent; in fact, the total lipid levels in skeletal muscle of animals whose diet was supplemented with DBH and Santoquin are only slightly elevated over those in the tocopherol-fed animals.

The results indicate that the effect of vitamin E on cholesterol and lipid levels of skeletal muscle and plasma is not that of a non-specific *in vivo* antioxidant but rather that either vitamin E is a highly specific antioxidant or that

TABLE VIII

Reproductive Performance of Female Rats Fed Unheated Soybean Oil and Heated Soybean Oil with and without Supplements of Linoleate or α-Tocopherol

Category	Females Bred (no.)	Litters Cast (%)	Litters (at birth)		Litters (at 3 days)		Litters (at 21 days)*		Mortality (%)	
			Total (no.)	Litters (no.)	Total (no.)	Average Weight (gm./rat)	Total (no.)	Average Weight (gm./rat)	0–3 Days	4–21 Days
Soybean oil	15	100	159	10.6	141	7.3	96	33.4	11	2
Soybean oil (LP)†	15	93	141	10.1	121	6.9	88	32.9	16	15
Soybean oil (HP)‡	15	80	110	9.2	72	6.8	59	34.6	34	0
Soybean oil (HP) plus linoleate	15	87	131	10.1	120	6.7	69	38.4	8	18
Soybean oil (HP) plus α-tocopherol	15	100	176	11.7	157	7.3	95	36.5	11	10

* Litters were cut to seven at the three-day period.
† Heated at 610°F. for seventy minutes.
‡ Heated at 610°F. for one hundred minutes.

it exerts its effect through some enzymatic mechanism in which non-tocopherol antioxidants are inactive.

It is generally accepted that the toxicity resulting from drastic heat treatment of unsaturated oils is a function of their polyunsaturated fatty acid content.[18–20] Interferences with nutrition which have resulted from the ingestion of these oils by various species of animals have been alleviated by administering fresh oil[21] and pyridoxine[22] supplements. In recent experiments[23] with fats and oils heated to 610°F. for periods of seventy and one hundred minutes, the only evidence of nutritional insufficiency was observed in a soybean oil which had been heated for the longer period of time. In animals fed this heated soybean oil, interference with reproductive performance was observed (Table VIII). The use of tocopherol as a supplement to the diet of these animals resulted in improved breeding, gestation and lactation. No changes in cholesterol metabolism were observed or were there any interferences with other nutritional indices studied such as growth, consumption of food, ability to digest the food or longevity. It was concluded that the amount of vitamin E necessary to regulate cholesterol levels in tissue is much less than that required for satisfactory reproductive performance in the rat, and that the vitamin E originally present in the oils was able to protect the polyunsaturated fatty acids from destruction during the heating process.

CONCLUSIONS

Although the requirement for vitamin E based on its protection against sterility and development of muscular dystrophy in animals has not been proved in man, the tocopherols have been accepted as essential for human nutrition. Tocopherols are the principal antioxidants in the body. Although there are some types of metabolic reactions in which tocopherol can be replaced by other antioxidants, there is no question that in some instances the requirement for tocopherol is specific. Antioxidants other than tocopherol may act to spare vitamin E for the metabolic processes for which it is essential. The activity of vitamin E in regulating cholesterol levels in various tissues of the body is probably due in part to its activity as an *in vivo* antioxidant but probably is due also to its activity in certain enzymatic processes.

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Vitamin E and Lipid Metabolism in Man

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THE PRESENT Elgin project, designed to investigate the tocopherol needs of man, is now in its sixth year. Some previously reported general conclusions[1–4] can be summarized as follows: (1) Tocopherol requirements are a function of the amounts of certain peroxidizable lipids in the diet and in the tissues. (2) The fatty acid composition of the lipids in all the tissues tested to date can be altered, within limits, by varying the fatty acid composition of the diet. (3) Linoleic acid appears to be the most significant oxidizable lipid component in diets of normal human beings. Other easily peroxidized fatty acids, such as arachidonic, and some of the higher molecular weight unsaturates in fish oils may also be important. (4) When peroxidizable fatty acids in the diet are low, the need for tocopherol decreases to low levels, but past dietary habits which have affected tissue composition must be taken into consideration in evaluating the need for tocopherol.

The organization and methods of the present Elgin project were described in detail previously.[1] Briefly, thirty-eight male subjects were divided into three groups: Group B (nineteen subjects) received a daily basal diet which contained approximately 2 mg. of tocopherol; group BE (nine subjects) received the same basal diet plus a supplement of 15 mg. of d-α-tocopherol acetate; and group HD (ten subjects) received the hospital diet, *ad libitum*. This controlled dietary regimen was started in October 1953 and continued to the present time. The basal diet provided 2,200 calories, 47 gm. protein and 60 gm. of fat of which 30 gm. was lard† which had been distilled *in vacuo* to remove most of the tocopherol. In April 1956, after two and a half years the lard in the basal diet was replaced by 30 gm. of stripped corn oil to increase the ingestion of linoleic acid. Nine months later (January 1957) the corn oil level of the basal diet was increased to 60 gm. per day. All corn oil used in the human studies was first distilled *in vacuo* to remove most of the tocopherol and aerated at 95°C. to a peroxide number of 50 to remove most of the residual tocopherols. (About two to three hours of heating was necessary to achieve this.) The lard used contained about 11 per cent linoleic acid, and the corn oil contained about 55 per cent linoleic acid.

EFFECTS OF TOCOPHEROL DEPLETION

Figure 1 compares the average tocopherol levels of the subjects in group B (unsupplemented basal diet) with the average peroxide hemolysis data which were obtained simultaneously. For each level of linoleic acid fed, it took approximately one to two years for the tocopherol levels to reach a plateau. However, the peroxide hemolysis reached maximum levels with the subjects on the lard diet within twenty-eight months, so that changes

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† Stripped lard, stripped corn oil and d-α-tocopherol compounds were supplied through the courtesy of Distillation Products Industries, Inc.; dl-α-tocopherol acetate was supplied by Hoffmann-La Roche, Inc.

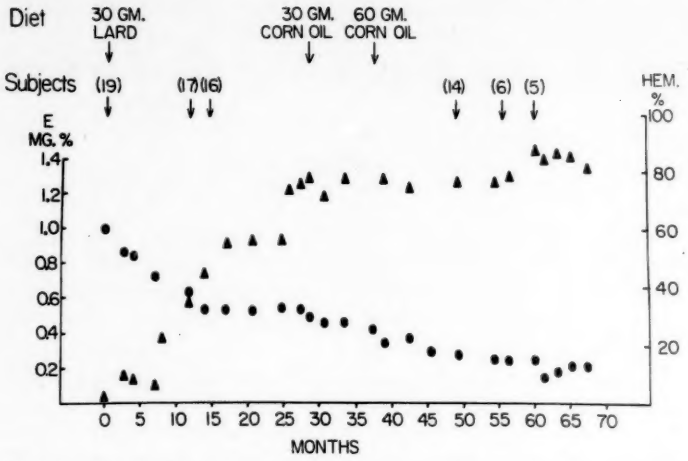

FIG. 1. Relationship of average plasma tocopherol to average peroxide hemolysis of subjects on depleted diet (group B) during five and a half years of dietary control. Numbers in parentheses indicate subjects remaining on depletion regimen. Plasma tocopherol—●; Hemolysis—▲.

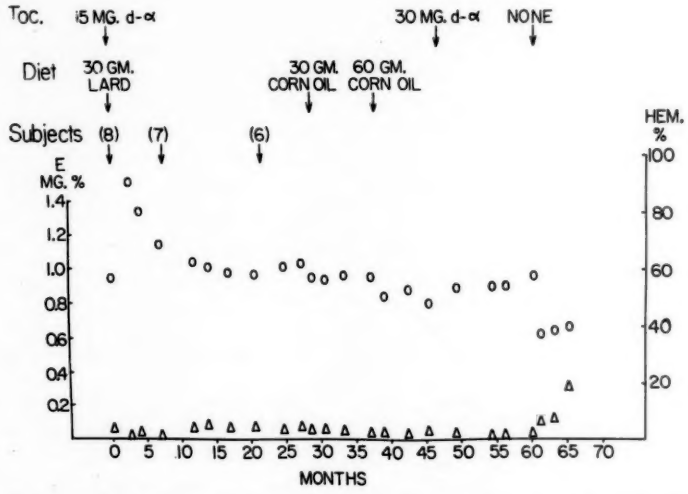

FIG. 2. Relationship of average plasma tocopherol to average peroxide hemolysis in subjects on supplemented diet (group BE) on basal diet. Plasma tocopherol—O; Hemolysis—Δ.

in the results of this test were slight after corn oil was added in the thirtieth month.

Figure 2 gives similar data for group BE. In this group, it took approximately twenty months on the lard diet for the plasma tocopherol level to return to its basal average after a sharp increase due to the initial supplementation. Each increase in the ingestion of linoleic acid lowered the tocopherol level of the plasma. At the end of four years the increase in the linoleic acid in the tissue of these subjects had caused an unexpected decrease in the

TABLE I
Effect of Tocopherol Withdrawal from Depleted Subjects Who Had Been Given Supplements

Subject	Tocopherol Supplements Given During First 53 Months of Project	Days Withdrawn from Last Tocopherol Supplement	Plasma Tocopherol (mg. %)	Peroxide Hemolysis (%)
BS 19	None for 13 months, 15 mg./day of d-α-tocopherol acetate for 23 months, and 30 mg./day of d-α-tocopherol acetate for 17 months	1	1.07	1
		2	0.86	1
		3	0.80	4
		4	0.78	4
		8	0.62	10
BS 18	None for 13 months, 15 mg./day of d-α-tocopherol acetate for 23 months, and 30 mg./day of d-α-tocopherol acetate for 17 months	1	1.21	1
		2	1.06	2
		3	0.94	3
		4	0.90	2
		8	0.76	20
BS 16	None for 46 months, and 105 mg./day of d-α-tocopherol acetate for 7 months	1	1.25	1
		2	1.06	2
		3	1.29	1
		4	0.98	2
		5	0.87	2
		8	0.81	7
BS 15	None for 46 months, and 140 mg./day of dl-α-tocopherol acetate for 7 months	1	0.96	1
		2	0.82	2
		3	0.70	2
		4	0.61	2
		5	0.65	2
		8	0.48	6

plasma tocopherol levels so that it was considered necessary to increase the d-α-tocopherol acetate supplement to 30 mg. or more per day. (The plasma tocopherol level of one subject dropped to 0.56 mg. per 100 ml.) At the 15 mg. level the tocopherol supplement was inadequate to counteract the effects of the linoleic acid in 60 gm. of corn oil. When the tocopherol supplement was discontinued, after the fifth year, there was a sudden drop in the plasma tocopherol level followed by a progressive increase in peroxide hemolysis over a period of five months.

EFFECTS OF TOCOPHEROL SUPPLEMENTATION

When blood for analysis is obtained within twenty-four hours after the ingestion of tocopherol by a patient on a supplemented diet, the plasma tocopherol level observed may be considerably higher than that in the blood samples obtained a day or so later. The extent of this difference was not apparent throughout the first four years of this study during which time a twenty-four–hour withdrawal period had been considered adequate to obtain basal levels of plasma tocopherol and peroxide hemolysis. Tables I and II give examples of these effects. Patients changed to a supplemented diet for forty months after they had been on a depleted diet for thirteen months showed a marked drop in plasma tocopherol and an increase in peroxide hemolysis only one week after tocopherol supplementation was stopped. The supplement had provided 15 mg. of tocopherol for twenty-three months and 30 mg. for seventeen months. This rebound effect, after five years of tocopherol supplementation, is also shown in Figure 2.

There is an important difference in the effects of withdrawal of tocopherol as shown in the comparison of peroxide hemolysis data from experimental subjects who had been receiving a depleted diet and then a supplemented diet (BS) with subjects who had not received a depleted diet (BE) at all (Tables I and II). Even after tocopherol supplements had been

TABLE II

Effect of Tocopherol Withdrawal from Subjects on Basal Diet Who Had Received Tocopherol As a Supplement

Subject	Tocopherol Supplements Given During First 53 Months of Project	Days Withdrawn from Last Tocopherol Supplement	Plasma Tocopherol (mg. %)	Peroxide Hemolysis (%)
BE 1	15 mg./day of d-α-tocopherol acetate for 46 months, and 30 mg./day of d-α-tocopherol acetate for 7 months	1	1.27	2
		2	1.14	2
		3	1.06	1
		4	1.00	1
		8	0.90	0
BE 2	15 mg./day of d-α-tocopherol acetate for 46 months, and 30 mg./day of d-α-tocopherol acetate for 7 months	1	0.92	1
		2	0.83	2
		3	0.78	1
		4	0.72	0
		8	0.58	2
BE 3	15 mg./day of d-α-tocopherol acetate for 46 months, and 30 mg./day of d-α-tocopherol acetate for 7 months	1	0.93	1
		2	0.76	0
		3	0.74	1
		4	0.72	1
		8	0.53	2
BE 5	15 mg./day of d-α-tocopherol acetate for 46 months, and 105 mg./day of d-α-tocopherol acetate for 7 months	1	1.47	2
		2	1.36	1
		3	1.29	1
		4	1.26	2
		8	0.94	1
BE 6	15 mg./day of d-α-tocopherol acetate for 46 months, and 140 mg./day of dl-α-tocopherol acetate for 7 months	1	1.16	2
		2	0.98	2
		3	0.92	2
		4	0.88	2
		8	0.78	5

given for forty months, the peroxide hemolysis went as high as 20 per cent when the supplement was withdrawn for only one week (BS 18). All these subjects received the same basal diet, which at this point contained 60 gm. of stripped corn oil. This phenomenon has now been confirmed by additional data on other subjects.

The effects of giving tocopherol supplements to subjects who had been receiving a depleted diet for fifty-four months are shown in Table III. These demonstrate the difficulty of returning the peroxide hemolysis to normal after depletion. Even after a supplement of either 15 mg. of d-α-tocopherol or 20 mg. of dl-α-tocopherol had been given for 138 days, the peroxide hemolysis was only partially reversed. We can only speculate as to whether this is due to the presence of increased concentrations of linoleic acid or to traces of peroxidized linoleic acid products which catalyze the *in vitro* reaction with hydrogen peroxide. This calls to mind the difficulty of reversing the effects of tocopherol depletion in animal experiments. This phenomenon may also be related to observations, which are discussed in another paper, of high peroxide hemolyses in adult patients who have never participated in our controlled dietary program, in whom tissue accumulations of linoleic acid must be a function of so-called normal non-experimental diets.

Note that 60 mg. or more of tocopherol per day is apparently adequate at this level of linoleate ingestion.

TOCOPHEROL-LINOLEIC ACID RELATIONSHIPS IN CHICKS

The relative ease of controlling the production of encephalomalacia in chicks affords a

TABLE III

Effect of Tocopherol Supplements on Subjects Who Had Been on Basal Diet for 54 Months*

Subject	Tocopherol Supplement	Period of Supplement (days)	Plasma Tocopherol (mg. %)	Peroxide Hemolysis (%)
B 3	None	0	0.12	83
		3	0.24	80
		6	0.20	85
		13	0.26	86
		21	0.25	86
		50	0.20	76
		138	0.16	90
BS 10	7.5 mg./day d-α-tocopherol acetate	0	0.18	86
		3	0.34	82
		6	0.37	80
		13	0.45	82
		21	0.42	90
		50	0.52	85
		138	0.56	91
BS 14	10 mg./day dl-α-tocopherol acetate	0	0.22	67
		3	0.29	70
		6	0.40	78
		13	0.34	78
		21	0.28	80
		50	0.30	52
		138	0.29	79
BS 13	15 mg./day d-α-tocopherol acetate	0	0.46	75
		3	0.66	35
		6	0.80	40
		13	0.64	32
		21	0.70	10
		50	0.71	10
		138	0.72	34
BS 6	20 mg./day dl-α-tocopherol acetate	0	0.18	85
		3	0.38	79
		6	0.37	70
		13	0.45	22
		21	0.41	40
		50	0.53	20
		138	0.57	25
BS 7	60 mg./day d-α-tocopherol acetate	0	0.30	73
		3	1.13	0
		6	1.16	0
		13	1.22	0
		21	1.16	2
		50	1.24	2
		138	1.29	1
BS 11	80 mg./day dl-α-tocopherol acetate	0	0.29	85
		3	0.60	0
		6	0.56	12
		13	0.70	2
		21	0.68	4
		50	0.74	2
		138	0.89	3
BS-8	240 mg./day d-α-tocopherol acetate	0	0.30	85
		3	1.38	0
		6	1.49	0
		13	1.47	2
		21	1.24	1
		50	1.18	1
		138	1.27	3
BS 12	320 mg./day d-α-tocopherol acetate	0	0.31	87
		3	1.14	1
		6	1.25	1
		13	1.34	1
		21	0.95	2
		50	0.99	2
		138	1.05	2

* Experimental diet furnished 2 to 4 mg. of tocopherol per day, and contained varying amounts of linoleic acid as described in text. Supplement withdrawn for forty-four hours before obtaining blood to allow time for plasma clearance of recently ingested tocopherol.

TABLE IV

Effect of Varying Lipids on Incidence of Encephalomalacia

Dietary Lipid (no vitamin E)	Peroxide No.*	Iodine No. (per kg. diet)	"Diene" (per kg. diet†) gm.	Incidence
No lipid	—	0	0	0/10
10% cod liver oil	45	160	2	0/18
10% oleic acid	—	89	2.2	0/9
15% butter	0	63	2.3	0/16
20% coconut oil	1	20	3.6	0/10
15% linseed oil	120	274	18‡	0/10
20% olive oil	60	178	23	0/19
4% stripped lard	28	28	5	0/10
15% stripped lard	28	92	17	10/20
20% stripped lard	28	128	22	15/21
4% stripped corn oil	58	50	22	48/70

* Peroxide number after aeration 95°c. to eliminate tocopherol.

† Diene content by alkaline isomerization and ultraviolet absorption.

‡ Linseed oil provided 81 gm. of linolenic acid per kg. diet.

splendid tool for assaying the relationship of tocopherol to variations in linoleic acid concentrations. The diets used have been described.[5] Table IV shows that the incidence of encephalomalacia in chicks can be related to the amounts of linoleic acid in the dietary fat. But there are exceptions, which we have published elsewhere,[5] to show that this relationship is not clear-cut. For example,[5] whereas 2 per cent corn oil will provoke encephalomalacia in seven of forty chicks, the addition of 10 per cent coconut oil to 2 per cent corn oil will increase the incidence to forty of fifty-one chicks. We have also shown[6] that the addition of 8 per cent of either linseed oil or cod liver oil will reduce the incidence of encephalomalacia markedly. Furthermore, by adding different fatty acids to a basal diet that contained 2 per cent corn oil, it was observed

TABLE V
Effects of Addition of Olive Oil and Linseed Oil on Chick Encephalomalacia

Dietary Lipids (treated to remove tocopherols)	Incidence
3% safflower oil	7/11
3% safflower oil + 12% olive oil	3/10
3% safflower oil + 12% linseed oil	0/11
5% safflower oil	6/10
5% safflower oil + 12% olive oil	6/10
5% safflower oil + 12% linseed oil	0/11

TABLE VI
Variations in Tocopherol and Corn Oil Intake on Incidence of Encephalomalacia

% Corn Oil in Diet (PN 50)*	mg d-α-Tocopherol Acetate per Week				
	0.0	0.2	0.5	1	2
0	0/10	0/15	0/9	0/15	0/5
1	1/10	0/10	0/9	0/9	—
2	2/6	1/6	—	0/6	0/6
2.5	4/10	2/10	0/9	0/9	—
4	14/19	10/19	2/10	2/18	0/7
8	8/8	4/7	—	1/8	0/7

* Peroxide number = 50.

that the addition of either lauric or myristic acid will increase the incidence of encephalomalacia; palmitic and stearic acid have little, if any, effect, and oleic acid has a protective effect. It appears from other data obtained with linseed oil[3,5,6] that linolenic acid has a protective effect on encephalomalacia in chicks (but not, it should be added, on chick oxidative diathesis[7]).

One possible explanation for these differences in producing cerebral lesions when tocopherol is absent might be that the saturated fatty acids are used for calories, preferentially, leaving the residue of linoleic acid to exert a larger "toxic" effect, whereas the unsaturated fatty acids may, for a time, compete for the available oxygen and, like antioxidants, prevent the peroxidation of linoleic acid. The fact that linseed oil is more protective than olive oil fits well with the fact that linolenic acid, the main component of linseed oil, is oxidized more readily than is oleic acid, which is the main component of olive oil. The effect of adding olive oil and linseed oil on the encephalomalacia potential of safflower oil is given in Table v in which the relative "protective" effect of these two oils is illustrated.

The direct relationship between the inges-

TABLE VII
Fatty Acid Compositions of Cerebellar Lipids from Chicks on Diets High and Low in Linoleic Acid

Fatty Acid	4% Stripped Corn Oil Diet (6)*		4% Fresh Corn Oil Diet (5)		10% Cod Liver Oil Diet (4)		10% Oleic Acid Diet (4)		Fat-Free Diet (4)	
	%	S.D.	%	S.D.	%	S.D.	%	S.D.	%	S.D.
Lauric	0.3	± 0.2	0.5	± 0.2	0.1	± 0.1	0.2	± 0.1	0.3	± 0.1
Myristic	1.0	± 0.7	2.8	± 0.6	0.6	± 0.3	0.6	± 0.2	1.4	± 0.5
Palmitoleic	1.3	± 0.2	1.7	± 0.5	1.4	± 0.6	2.1	± 0.4	1.7	± 0.2
Palmitic	37.5	± 4.0	32.4	± 4.2	39.5	± 6.6	28.6	± 5.1	33.4	± 4.0
Linoleic	1.9	± 1.0	2.0	± 0.8	0.5	± 0.2	0.4	± 0.1	0.9	± 0.3
Oleic	26.9	± 3.9	21.9	± 2.4	33.6	± 2.2	40.6	± 4.0	33.1	± 5.7
Stearic	18.4	± 1.5	16.2	± 2.3	17.3	± 2.4	16.1	± 3.1	15.3	± 2.0
C_{20} pentaene and tetraene	3.8	± 3.1	3.0	± 1.1	1.3	± 1.0	2.0	± 0.9	1.1	± 0.8
C_{20} triene	0.9	± 0.5	2.2	± 1.1	0.3	± 0.1	0.7	± 0.3	1.0	± 0.4
C_{22} hexaene	1.7	± 2.2	1.7	± 0.9	2.3	± 2.2	1.5	± 1.2	0.8	± 0.7
C_{22} pentaene	1.4	± 1.7	1.0	± 0.7	0.7	± 0.5	0.4	± 0.2	0.3	± 0.3
Others	4.8		14.6		2.4		6.8		10.7	
Linoleic to oleic ratio	0.072	± 0.044	0.095	± 0.041	0.014	± 0.005	0.010	± 0.004	0.029	± 0.012

* Number of chicks.

TABLE VIII
Fatty Acid Compositions of Human Erythrocytes

Fatty Acid	Subjects on Institutional Diet (8)* % S.D.	Subjects on Corn Oil Diet for 4 Years (19) % S.D.	Subjects on Corn Oil for 4 Years and Coconut Oil for 80 Days (4) % S.D.
Lauric	0.4 ± 0.2	0.5 ± 0.5	0.3 ± 0.1
Myristic	0.7 ± 0.3	0.8 ± 0.6	1.3 ± 0.2
Palmitoleic	0.6 ± 0.4	0.5 ± 0.2	1.0 ± 0.5
Palmitic	30.2 ± 3.1	27.2 ± 2.6	29.6 ± 2.0
Linoleic	8.3 ± 1.5	15.3 ± 1.6	13.0 ± 1.8
Oleic	20.4 ± 3.3	17.1 ± 1.5	18.1 ± 1.2
Stearic	20.4 ± 1.8	19.2 ± 1.4	21.0 ± 0.5
C_{20} pentaene and tetraene	10.4 ± 3.6	10.0 ± 3.2	8.3 ± 1.3
C_{20} triene	1.9 ± 0.7	2.0 ± 0.8	1.8 ± 0.4
C_{22} hexaene	1.2 ± 0.7	1.1 ± 2.3	0.3 ± 0.2
C_{22} pentaene	2.1 ± 1.0	1.6 ± 1.0	2.2 ± 0.4
Others	3.4	4.7	3.1
Linoleic to oleic ratio	0.42 ± 0.10	0.90 ± 0.13	0.73 ± 0.14

* Number of subjects.

tion of linoleic acid-containing fat and the amount of tocopherol required to prevent encephalomalacia in chicks is illustrated in Table VI.

Encephalomalacia in chicks is accompanied by a cerebellitis in which the hemorrhages, cerebellar petechiae and necrotic lesions are grossly visible. The histological preparations are described by Bailey[5] as "hemorrhages which involve both the molecular and granular layers of the cerebellum. When the hemorrhages are well developed the Purkinje cells have entirely disappeared."

FATTY ACID IN CEREBELLA OF CHICKS

In an attempt to correlate the incidence of encephalomalacia in chicks with the amounts of linoleic acid present in the brain, the individual cerebella from brains of one month old chicks were analyzed for fatty acids using micro gas chromatographic technics.[8–11] Table VII summarizes the results of such analyses. Increased amounts of linoleic acid were observed in the cerebella of chicks which were fed diets containing higher levels of linoleic acid. Interestingly enough, the cerebella of the chicks on fat-free diets contained moderate levels of linoleic acid. Although it is possible that the chick can synthesize limited quantities of this compound, the amount contributed by the egg may be important and cannot be evaluated accurately, since the linoleic acid content in eggs will vary depending upon the diet fed to the hen.

FATTY ACIDS IN HUMAN ERYTHROCYTES

In order to ascertain the relationship between the level of linoleic acid in the blood cells and the relative irreversibility by tocopherol of the peroxide hemolysis test results, the fatty acids of the erythrocytes of the experimental subjects were estimated by gas chromatographic methods.[11] Such changes were first reported[1] about four years ago, using cruder methods. The levels of linoleic acid were considerably higher in the erythrocytes obtained from subjects ingesting corn oil than from subjects who ate the regular hospital diet, *ad libitum* (Table VIII). From the data obtained, one cannot distinguish between subjects who had been on a depleted diet or a supplemented diet, nor can one distinguish between subjects who received fresh commercial corn oil instead of oxidized stripped corn oil. However, in every subject who had received a corn oil ration for several years the linoleic acid concentration in his erythrocytes was higher than that in any patient who had not

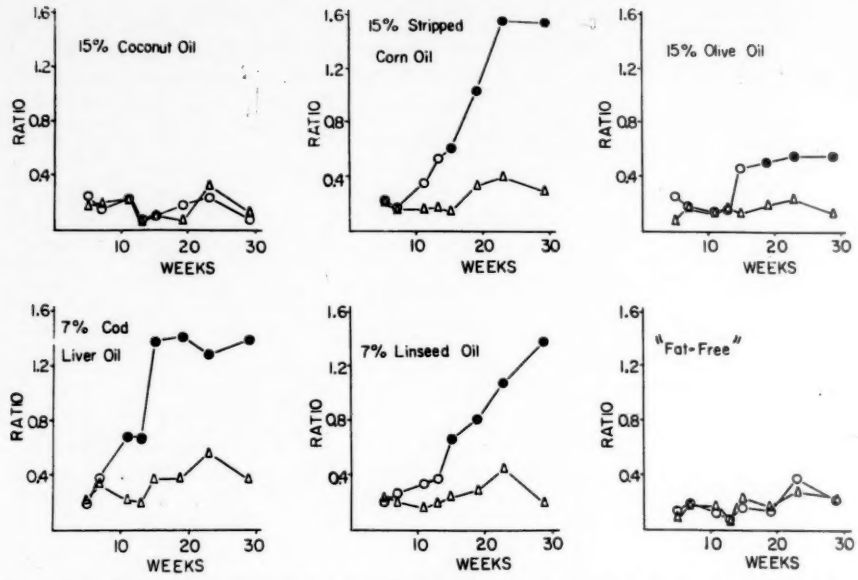

FIG. 3. Creatine: creatinine ratios in tocopherol-deficient rats. Relationship of type of lipid in diet to the production of creatinuria. Note that unless the diet provided a liberal amount of peroxidizable lipids there was no effect on the relative amount of creatine (ratio) excreted showing that tocopherol was not required to prevent creatinuria when ingestion of peroxidizable lipids was low. Circles represent animals that received no tocopherol and triangles designate animals that were given 4 mg. d-α-tocopherol acetate per week, orally. Statistically significant points in curves as determined by the t test where probability was better than 95 per cent are indicated by the filled circles. The diets contained 25 per cent casein and the amounts of fat indicated. The so-called fat-free diet contained 0.2 per cent stripped corn oil to provide the trace of essential fatty acids necessary. Additional details on this experiment will be published elsewhere. Effect of different lipids in diet (Δ = E+, O = E−, ● = Significant).

received the experimental diet. Recently, a group of subjects who had been on a corn oil diet for several years were placed on a coconut oil diet instead. After several months, little change has been noticed in the amounts of linoleic acid, and although the elapsed time is too short to make more than a preliminary statement, it is apparent that this accumulation of linoleic acid by erythrocytes is not easily reversed in the adult. When all the facts are in, these observations may have to be correlated with the data obtained on the effects of coconut oil in aggravating the encephalomalacia potential of small amounts of corn oil in chicks.

CEREBELLAR LESIONS IN AN INFANT

The difficulty of obtaining fresh brain tissue from man makes any attempt to correlate chick encephalomalacia with human requirements for tocopherol and linoleic acid most onerous. However, the histologic specificity of the cerebellitis observed in chick encephalomalacia makes any similar histopathologic observation in man of special significance.[12] Such lesions were found by Bailey during the course of routine pathologic analyses of the brain of a fourteen month old infant who had been brought to the Illinois Research and Educational Hospital as a feeding problem that was secondary to the growth of an abdominal tumor. The child's brain, like those of the chicks', appeared normal except for the cerebellum which was the site of widespread hemorrhages, proliferation of the capillaries and absence of Purkinje cells. It should be emphasized that there were no lesions in the brain stem or other parts of brain so this observation

should not be confused with Wernicke's syndrome.

On following up this interesting observation, it was learned that an attempt had been made to ameliorate the severe malnutrition of this infant by intravenous feeding which included a commercial cottonseed oil preparation that provided about 23 gm. of linoleic acid per day for nineteen days before its death or more than ten times the relative ratio of linoleic acid to calories needed to produce encephalomalacia in the chick. The vitamin supplements which had been given this child did not contain tocopherol. To our knowledge this is the first time that such a lesion has been noted in a human brain. Some years ago there was some controversy on the possible efficacy of vitamin E in the prevention or cure of retrolental fibroplasia in premature infants by Owens and Owens[13] and some reports on the prevention of cerebromeningeal hemorrhages in premature infants by Minkowski.[14] Our present information, which may place the concentrations of linoleic acid in the lipoproteins in a position of primary importance in tocopherol deficiencies, may stimulate the reinvestigation of these problems.

TOCOPHEROL-LINOLEATE RELATIONSHIP IN THE RAT

Restrictions of space do not permit presentation of more than a small fraction of the data accumulated during the past six years, but some pertinent data from studies on rats must be included. The rat, like man, may differ from the chick in that it must have a certain amount of tocopherol in its tissues to bring it through the weanling stage. The chick can come from an egg which may be very low in tocopherol and can start life as a tocopherol-deficient bird. Accordingly, different manifestations of disease are noted in the rat which may be associated with the longer time interval necessary to eliminate larger tocopherol storage.

Figure 3 summarizes the results of a study in which the appearance of nutritional muscular dystrophy in the rat was the objective analyzed. All lipids used were stripped of tocopherol as previously described. Note that on a 15 per cent coconut oil diet and on a "fat-free" diet (which actually provided 0.2 per cent stripped corn oil to supply the necessary essential fatty acids), the twenty-four–hour urinary excretions of creatine were not greater than in the control animals receiving 4 mg. of d-α-tocopherol acetate per week. Only a moderate, but significant, amount of pathologic lesions was noted in rats receiving 15 per cent olive oil; whereas 7 per cent cod liver oil, 15 per cent corn oil and 7 per cent linseed oil produced markedly increased excretions of creatine. The data from these rat experiments cannot be directly related to the amounts of linoleic acid in the diet; however, they do confirm past observations that other polyunsaturated fatty acids can be involved in the production of nutritional muscular dystrophy. (Supporting data on effects of ingestion of different lipids on changing the fatty acid composition of muscle and brain mitochondria will be published elsewhere.) The differences between these results and those in chicks may be partially explained by noting that all encephalomalacia data are recorded after only four weeks of life, whereas in the rat it took more than ten weeks after weaning for abnormalities to develop. However, as far as human diets are concerned, high linoleic acid concentrations are more likely to be encountered than are high concentrations of arachidonic or other polyunsaturated fatty acids.

COMMENTS

It is now apparent that any evaluation of human requirements for tocopherol must be a function of the amount of polyunsaturates or, to be practical, the amounts of linoleic acid in the diet. That this is not a simple relationship has been demonstrated by experiments on the modifying effects of other fatty acids on linoleic acid in the production of lesions in chicks and rats. This is further complicated by the observations that once the body lipoproteins have accumulated linoleates and have been exposed to peroxidation, it may take abnormally large amounts of tocopherol to inhibit further peroxidation.

Much of present information on human requirements for tocopherol rests on interpre-

tations of the peroxide hemolysis test. I have never considered[1] this test to be more than an indication of the rate at which erythrocyte fatty acids can be oxidized. In our work with adults having satisfactory protein reserves, no correlation with catalase concentrations, or erythrocyte structure, has been found, whereas the content of oxidizable lipids in the red blood cell did vary directly with the incidence of increased peroxide hemolysis. There may be an objection to attaching too much significance to this *in vitro* test as increases in linoleic acid in the absence of adequate tocopherol should cause a more rapid oxidative breakdown of tissues *in vitro*, but it is this simple relationship of the amount of peroxidizable matter vs. the amount of antioxidant that remains the core of the problem and no better means of estimating this relationship is at hand. Thus, the observations by Gordon et al.[15,16] and György et al.[17] on increased peroxide hemolysis sensitivities in premature infants have been fortified by the present controlled study on adults.

With regard to the actual α-tocopherol requirements of adults, it would seem that as little as 5 mg. per day should be adequate for an individual with minimal stores of linoleic acid if he is on a diet that is low in unsaturated lipids. But it is also apparent that when the stores of linoleic acid have been increased by the prolonged ingestion of large amounts of vegetable oils, as much as 30 mg. per day or more may be required to minimize tissue lipid peroxidation. The fact that no special enzyme system has been proved to be involved in tocopherol deficiency in no way lessens the importance of the need for vitamin E. It should be obvious that the lipoprotein component of any enzyme system, especially if altered by linoleate ingestion, may require the protection by an antioxidant. In the search for specific enzymes that might be related to vitamin E deficiency, it seems that the simplest relationship based upon the ability of the lipids of the mitochondria to vary with diet has been overlooked. Once a lipoprotein has been peroxidized, this procedure cannot be reversed by adding tocopherol so that the criterion of reversibility of vitamin function as biochemical proof of the need for a vitamin may not be applicable in this case.

Within recent years, the increased ingestion of linoleic acid has been recommended to decrease the levels of plasma cholesterol. In this way, linoleic acid may be considered a pharmacological tool to achieve a specific clinical end; no one can possibly object to the further use and study of this procedure. However, one questions the propriety, in the absence of all the facts, of making radical changes in national dietary habits by publicly recommending increased consumption of unsaturated fats. Preliminary scientific evidence should not be interpreted to produce excesses. Recently published reports[18] show that the incidence of cerebral hemorrhages in Japan are twice that encountered in North America. The diets in Japan are rich in linoleic acid and the incidence of hypertension is also high. In comparing these statistics with the data presented in this paper no one should condemn the ingestion of moderate levels of linoleic acid. However, when large amounts of linoleic are recommended, an evaluation of the amounts of tocopherol ingested should also be made.

CONCLUSIONS

The lipid composition of the brain, the red blood cell and presumably of all other vital tissues, can be altered by dietary means.

Altering the levels of unsaturated lipids in the tissues by dietary means can produce pathological changes.

The levels of tocopherol required to protect the tissue lipids from peroxidation is a function of the amounts of peroxidizable lipids present.

Feeding lipids high in linoleic acid increases the need for tocopherol. Although most lipids high in linoleic acid are also high in tocopherol, preliminary evidence indicates that tissue tocopherol may be stored less efficiently than tissue linoleic acid, leaving a tocopherol deficit in situations in which linoleic acid consumption has been abnormally high.

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Nutritional Influences on the Metabolism of Bile Acids

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THE CONTRIBUTION of bile to the normal physiology of vertebrates has been appreciated, however imperfectly, since antiquity. The specific action of the bile salts as surface-active agents in promoting the emulsification and absorption of lipids has also been recognized for a long time. Many of the early observations, which suggested that bile was indispensable for the absorption of lipids and fat-soluble vitamins, were made in the clinic from the study of patients in whom the concentrations of bile salts in the intestine were reduced or absent as a result of diseases of the hepatobiliary tract.

One consistent finding in the patient with acute or chronic obstruction of the bile duct has been the elevation of the serum cholesterol and phospholipid levels. In view of the suspicion, later confirmed, that cholesterol cannot be absorbed from the gastrointestinal tract without bile salts, this observation must have been puzzling. The hypercholesteremia associated with obstruction of the bile duct, together with the structural similarity of the bile salts to cholesterol established by Wieland and others,[1] suggested that cholesterol might serve as a precursor for bile salt synthesis.

Bloch and associates[2] established that cholesterol can, in fact, serve as a precursor for cholic acid synthesis *in vivo*. Later studies[3] have quite firmly established that the bile salts are formed by oxidation of the three terminal carbons of the side chain of cholesterol together with isomerization and oxidation at certain sites on the steroid nucleus. In many higher vertebrates, and certainly in nearly all, if not all, mammals, the principal bile acids are 24-carbon compounds formed by oxidation of the terminal carbon, followed by β-oxidation and cleavage of the side chain between carbons 24 and 25. Although oxidative cleavage of the cholesterol side chain occurs much closer to the steroid nucleus in reactions occurring in the adrenals and gonads, there is no evidence that the side chain of the bile salts is further cleaved by mammalian tissues or by organisms normally present in the gastrointestinal tract. This resistance to further oxidation of bile acids may be related to the demonstration that changes in the ring structure precede the initial cleavage of the side chain or to the presence of the gamma methyl group in the bile acid side chain.[3] Another reasonable explanation is that the hepatic cell effectively keeps bile salts out of the systemic circulation, and the conjugating activity of the liver is so great that the carboxyl group is quickly masked.

It should be stated parenthetically that the sequence of changes involved in the conversion of cholesterol to bile acids has been worked out largely by studies of the disposition of various labeled hypothetical intermediates administered to the intact rat.[3] Attempts to describe *in vitro* systems that will perform the various steps have been much less successful. Cer-

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tainly the complete conversion of cholesterol to naturally occurring bile acids has not been worked out *in vitro*.

It has been shown in rats and in man that when labeled cholesterol is injected, most of the labeled material in the bile is present as bile acids. Furthermore, a significant portion, perhaps as much as one-half, of that cholesterol which is in the plasma or is readily exchangeable with the plasma cholesterol, is eventually excreted into the feces as bile acids.

Attempts to perform sterol balance studies in the same way nitrogen balance studies would be performed are without meaning. Not only do most body tissues have the capacity to synthesize cholesterol, but the rate of cholesterol synthesis is also influenced by the ingestion of certain sterols including cholesterol. Furthermore, a large number of different steroids are derived from cholesterol and ultimately excreted. It is, nevertheless, of considerable fundamental as well as practical importance to be able to measure the rates of conversion of cholesterol to its end products and the rates at which these products are excreted by the whole organism.

A significant quantity of cholesterol is converted to steroid hormones by the adrenals and gonads and excreted as such or in modified form into the urine, but most of the cholesterol is excreted either as neutral sterols or as bile acids in the feces. Both of these latter groups of compounds appear in the feces as large series almost certainly elaborated in large part by the flora of the large intestine. Many of the constituents of both series have been identified, but neither series has been completely defined.

It would seem then that in studying the various processes involved in elimination of cholesterol from the body, two broad types of reactions are involved: first, oxidation of the cholesterol molecule by the liver, gonads and adrenals; and second, the metabolism and ultimate excretion of these products of oxidation as well as of cholesterol itself and other neutral sterols. It is possible that factors which alter the rates of formation, cycling and excretion of bile acids may influence the concentration of cholesterol in tissues. However, it would be unwise to anticipate effects on serum cholesterol levels from measurements of bile acid excretion. A high rate of bile acid excretion would inevitably imply a high rate of precursor (cholesterol) synthesis (assuming no shift from other cholesterol pools). In the steady state, the size of the cholesterol pool from which the bile acids were formed could be quite independent of the rates of bile acid formation and excretion. It is, of course, regulation of the size of this pool of cholesterol with which so many investigators have been concerned (even though their efforts might be misguided).

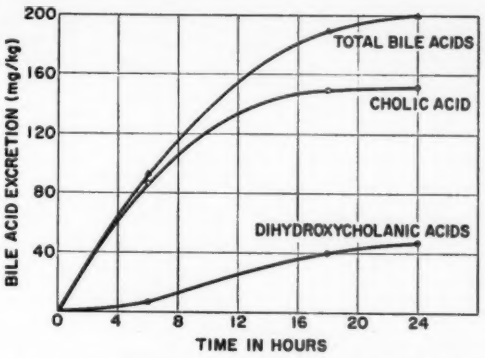

Fig. 1. The cumulative excretion of cholic acid, dihydroxycholanic (chenodeoxycholic) acid, and total bile acids by bile fistula from a rat previously fed Purina Laboratory Chow. From: PORTMAN, O. W. and MANN, G. V. *J. Biol. Chem.*, 213: 733, 1955.[4]

EXCRETION OF BILE ACIDS INTO THE BILE

Our initial attempts[4] to study the effects of diet on bile acid excretion were performed using rats with biliary fistulas which had been prefed the diets (while under study) for fifteen days prior to cannulation. Bile was then collected for twenty-four hours following cannulation and bile acid determinations were performed on the collected bile. In nearly all cases a characteristic pattern of excretion similar to that observed in Figure 1 was seen.

The initial collection of bile was rich in bile acids. With time the concentrations progressively decreased, although the absolute volume of bile was fairly well maintained. After twelve hours of continuous bile drainage, the quality of bile acids excreted per unit time became

quite small and the ratio of chenodeoxycholic acid to cholic acid excreted increased. Eriksson[5] found a similar pattern of excretion of bile acids during the first twenty-four hours after cannulation. He also observed that after this initial period the rate of bile acid excretion became extremely rapid, and on the second and following days after cannulation, the continuous rate of bile acid excretion was similar to that seen immediately after cannulation.

The earlier studies on the twenty-four hour excretion of bile acids by rats showed that the diet fed before cannulation had a significant influence on the quantity of bile acids excreted. The addition of cholesterol to a purified diet or to Purina Laboratory Chow resulted in a small but significant increase in the excretion of bile acids. However, the most striking difference in the bile acid excretion by different groups of rats was that observed between rats fed the purified diet based on sucrose and those fed Purina Laboratory Chow.[4,6] The latter group excreted three times more cholic acid and two times more total bile acids than the rats fed the purified diet. A number of dietary variations were made to explain the observed differences; however, the only changes made in the basal purified diet which caused an increase in bile acid excretion were the substitution of cornstarch for sucrose or the addition of fiber to the diet.

DIET AND FECAL EXCRETION OF BILE ACIDS

As previously noted, there are limitations to the use of rats with cannulas in the bile duct. It was more meaningful for our purposes to measure the fecal excretion of bile acids. Technical difficulties prevented our attempts to do this until a procedure for labeling bile acids was described by Bergström and associates.[7] Using labeled cholate, and the procedure outlined by Lindstedt and Norman[8] for calculation of bile acid turnover and pool size, certain of the diets discussed herein were re-evaluated.[9] Since the publication of the original report describing the calculation of bile acid turnover,[8] a later study by Lindstedt and Samuelsson[10] has described the calculation of turnover rates of coincident pools of bile acids, separate rates being associated with bile acids of the large intestine.

TABLE I

The Effect of Diet on the Daily Excretion of Cholic Acid and Metabolites and of β-Hydroxysterols in the Feces of Rats

Diet	Half-Life Cholic Acid (days)	Cholate Pool (mg./kg.)	Cholic Acid Excreted (mg./kg./day)	β-OH Sterols Excreted (mg./kg./day)
Chow	2.0	98.8	36.4	75.4
Sucrose	4.2	45 6	7.7	44.0
Starch	3.2	45.0	10.3	48.1
Sucrose + 20% fiber	1.4	45.3	23.4	29.9

NOTE: Chow indicates Purina Laboratory Chow. The purified diets contained (per cent) casein, 20; corn oil, 8; carbohydrate, 67.6; salts, 4; inositol, 0.1; choline, 0.2; *p*-aminobenzoic acid, 0.1; and adequate supplements of fat and water-soluble vitamins. From: PORTMAN, O. W. and MURPHY, P. *Arch. Biochem.*, 76: 367, 1958.[9]

N = Four rats per group.

Variations in the fecal excretion of bile acids were obtained by feeding different diets (Table I). In general, rats fed diets associated with greater quantities of bile acid excretion via biliary fistulas also showed greater rates of fecal excretion of bile acids.

Although there was some suggestion that the large excretion of bile acids from biliary fistulas or from feces of rats fed the Purina chow diet might be related to the presence of complex carbohydrate or fiber, a further attempt was made to ascertain which component of the natural diet contained the cholepoetic activity. Five diets were evaluated: (1) the sucrose-containing purified diet; (2) purified diet plus a fraction representing a continuous ethanol extract of Purina Laboratory Chow; (3) purified diet plus the non-saponifiable component of lipids from Purina chow; (4) extracted chow plus added vitamins and fat; and (5) unmodified Purina chow. The results (Table II) demonstrate that the cholepoetic activity in Purina chow is not associated with any sterol or other lipid. The bile acid excretion of the rats fed Purina chow or extracted

TABLE II

The Effect of Diet on the Biological Half-Life of Cholic Acid, on the Size of the Cholate Pool and on the Total Excretion of Cholate and Metabolites in the Feces of Rats

Diet	Half-Life Cholic Acid (days)	Cholate Pool (mg./kg.)	Cholic Acid Excreted (mg./kg./day)
Chow	2.0	100.1	34.6
Extracted chow	2.2	97.4	30.5
Sucrose + chow lipid	2.8	49.9	12.3
Sucrose + chow non-saponifiables	3.7	63.9	11.9
Sucrose	4.2	61.6	10.3

NOTE: Chow is Purina Laboratory Chow. Extracted chow had been continuously extracted with ethanol; fat and water-soluble vitamins and corn oil in the same concentrations as those present in the sucrose purified diet were added to the extracted chow.
N = Eight rats per group.

Purina chow reconstituted with vitamins and fat were nearly identical.

Commercial animal feeds, such as Purina Laboratory Chow, include a mixture of various grains. Therefore, the cholepoetic effect of diets containing a single grain was evaluated. The following diets were evaluated: (1) chow; (2) expressed corn germ diet; (3) solvent extracted corn germ diet; (4) purified diet containing sucrose; and (5) purified diet with 80 per cent lactose and 20 per cent sucrose (Table III). In rats fed diets containing corn germ bile acid excretion was greater than in those fed diets containing the simpler sugars and without added fiber. It is interesting that in rats fed the lactose-containing diet the half-life of injected radiocholate was longer than in those fed the sucrose-containing diet.

Since Lindstedt and Norman[8] demonstrated that the administration of intestinal chemotherapeutic agents to rats resulted in greatly increased biological half-lives of radiocholate, it seemed possible that the differences in bile acid turnover seen in groups of rats fed different diets could be explained as a result of variations in intestinal flora. Therefore, measurements of bile acid excretion were made on groups of rats fed various diets with and without added succinylsulfathiazole (Sulfasuxidine®) at 0.5 per cent of the diet by weight.

TABLE III

The Effect of Diet on the Biological Half-Life of Cholic Acid, on the Size of the Cholate Pool and on the Total Excretion of Cholate and Metabolites in the Feces of Rats

Diet	Half-Life Cholic Acid (days)	Cholate Pool (mg./kg.)	Cholic Acid Excreted (mg./kg./day)
Chow	2.2	129.0	40.4
Corn germ expressed	3.1	132.0	29.4
Corn germ extracted	2.2	154.0	48.3
Sucrose	4.2	52.0	8.5
Lactose	6 5	58.4	6.2

NOTE: Chow indicates Purina Laboratory Chow. Expressed corn germ had been commercially processed for the removal of corn oil. Extracted corn germ had been subjected to continuous solvent extraction. The sucrose and lactose diets had the composition indicated in Table I. The corn germ diets were reconstituted with corn oil and casein to make them equivalent to the purified diets in nitrogen and lipid content.
N = Six rats per group.

TABLE IV

The Effect of Diet and Intestinal Chemotherapeutic Agents on the Biological Half-Life of Cholic Acid, on the Size of the Cholate Pool and on the Total Excretion of Cholate and Metabolites in the Feces of Rats

Diet	Half-Life-Cholic Acid (days)	Cholate Pool (mg./kg.)	Cholic Acid Excreted (mg./kg./day)
Chow	1.9	100.0	36.4
Chow + sulfa	6.6	164.5	17.2
Sucrose	5.6	59.1	7.3
Sucrose + sulfa	11.4	76.3	4.6
Sucrose + 20% fiber	1.6	54.8	23.6
Sucrose + 20% fiber + sulfa	5.4	62.9	8.0

NOTE: Sulfa indicates a concentration of 0.5 per cent Sulfasuxidine® (Merck-succinylsulfathiazole) in the diet. See Table I for other diet designations
N = Four rats per group.

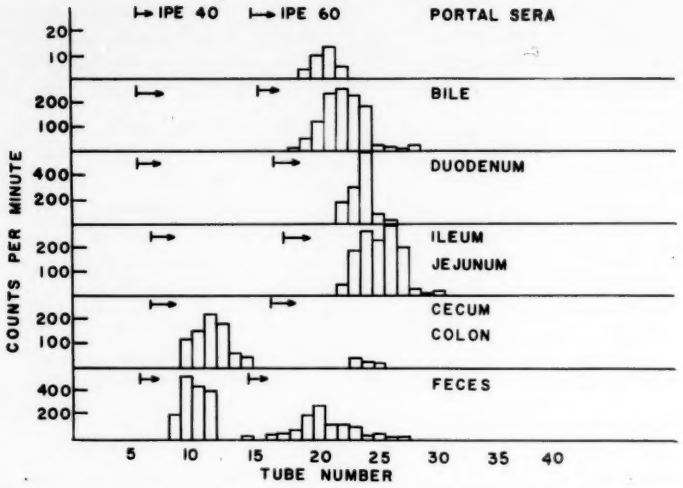

Fig. 2. The chromatographic[18] distribution of radioactivity in hydrolyzed samples of bile, portal serums, feces and contents of various segments of the gastrointestinal tract of a rat on a diet of Purina Chow. The rat had been given an injection one day previously of 2 mg. (4.4 μc.) of cholic acid-24-C^{14} (pH 7.4 phosphate buffer). The IPE40 region would include the dihydroxycholanic acids and the IPE60 region the trihydroxycholanic acids. Paper chromatographic analyses of the unhydrolyzed samples indicated that the labeled compounds in the feces and cecum-colon were unconjugated while the labeled compounds in the other four samples were conjugated with taurine. From: PORTMAN, O. W. *Arch. Biochem.*, 78: 125, 1958.[11]

The various dietary groups had prolonged biological half-lives for radiocholate when this bacteriostatic agent was used (Table IV). The pool of cholate expanded in size in each case, but there was a net decrease in excretion of cholic acid and its metabolites. The addition of Sulfasuxidine resulted in a decrease in the differences in bile acid excretion related to diet.

MODIFICATION OF BILE ACIDS IN THE GASTROINTESTINAL TRACT

An understanding of the way in which diet influences bile acid excretion was sought by a study of the chemical changes in the bile acids in the gastrointestinal tract. Our consistent findings[9,11] have been that after injection of trace doses of radiocholate to rats, the labeled material found in the bile, small intestine and portal blood was almost entirely unmodified cholic acid conjugated with taurine. In the cecum, large intestine and feces the labeled material was present almost entirely as unconjugated derivatives of cholic acid. As indicated in Figure 2, which shows radiochromatographs of material from various sites, there are two broad peaks of radioactivity in the separation of labeled material from feces of rats fed Purina Laboratory Chow. The peak in the IPE40 (or dihydroxycholanic acid) region was invariably present regardless of the nature of the diet, and this has been shown to be largely deoxycholic acid. The second broad peak in the IPE60 region was present in significant quantities only in those groups of rats with the more rapid rates of excretion of metabolites of radiocholate. On the basis of the studies of Norman and Sjövall,[12] it would be logical to assume that the band in the IPE60 region is a mixture of cholate, 7-ketodeoxycholate and, perhaps, members of the hyocholate series.* Both cholate and 7-ketodeoxycholate are microbiological precursors of deoxy-

* Although existing evidence indicates that the hyocholate series is derived from chenodeoxycholic acid.

TABLE V

The Effect of Diet and Fasting on the Distribution of Radioactive Materials in the Intestinal Contents and Wall, Forty-eight Hours After the Intraperitoneal Injection of Cholate-24-C^{14}

Diet	Small Intestinal Contents (%)	Small Intestinal Wall (%)	Large Intestinal Contents (%)
Sucrose.....	73.9	9.1	17.0
Sucrose (fast)...	58.1	15.2	26.7
Chow.....	85.6	7.8	6 6
Chow (fast)....	57.0	11.7	31.2

NOTE: The fasted rats were not fed from twenty-four hours prior to injection until the time of sacrificing. Less than 0.5 per cent of the activity was found in the wall of the large intestine.

N = Three rats per group.

cholate in the rat. It seemed to be a reasonable working hypothesis[12] that the more rapid excretion of bile acids in animals fed diets of greater bulk might simply be related to greater gastrointestinal motility with a larger proportion of the unmodified bile acids reaching the cecum, or area of bile acid modification and slow reabsorption, in each enterohepatic cycling.

DIET AND DISTRIBUTION OF BILE ACIDS IN THE GASTROINTESTINAL TRACT

This latter hypothesis, that diet may influence the rate of excretion of bile acids by changing the proportion of bile acids found in the small intestine (or area of rapid bile acid absorption) to those in the large intestine (or area of slow absorption), was tested by giving an injection of a test dose of radiocholate to rats fed different diets and measuring the distribution of radioactivity in the small intestinal contents, small intestinal wall, large intestinal contents and large intestinal wall at various periods after injection. Five diets were studied: (1) a sucrose-containing diet; (2) a sucrose diet with 5 per cent fiber; (3) a sucrose diet with 20 per cent fiber; (4) a cornstarch diet; and (5) a Purina Chow diet. In addition, groups of rats that were previously fed the sucrose or Purina Chow diets were fasted from twenty-four hours before injection until the time they were killed. There was remarkably little difference in the distribution of radioactivity in the gastrointestinal tract regardless of the diet fed or whether rats were killed twenty-four or forty-eight hours after the injection of radiocholate (Table v). The proportion of labeled bile acids found in the large intestine of the fasted rats was greater than that in the control animals, which were fed. This latter observation is in contrast to that of Norman and Sjövall.[12] A negligible amount of radioactivity was found in the wall of the large intestine.

ABSORPTION OF BILE ACIDS

Although bile acids are more slowly absorbed from the large intestine than from the small intestine,[10] there is little evidence to indicate whether the bacterial modification products of cholate are less readily absorbed from the intestine than is cholate. The rates of biliary excretion of test doses of cholate-C^{14} and deoxycholate-C^{14} administered intraduodenally to rats are indicated in Figure 3. Rats treated with radiodeoxycholate excreted considerably less of the test dose than those given radiocholate. Chromatography of the biliary bile acids indicated that much of the deoxycholate had not been converted to cholate in the single passage through the liver. Since deoxycholate is not prevalent in rat bile, it appears that the test doses were unphysiologically large. The more pertinent site to consider when evaluating rates of absorption of the bile acids would be the cecum, where the various conversion products of cholate are formed *in vivo;* however, the cecum would have to be kept sterile if meaningful results were to be obtained. Studies[4,9,11] which have been carried out using taurocholate-S^{35} and cholate-C^{14} indicated that the most prevalent bile acid absorbed into the portal circulation of the rat is taurocholate.

Attempts to study the absorption of bile acids by an isolated loop of the intestine have not been successful in our hands. We have, therefore, resorted to measurements of the degree of elevation of the serum cholesterol level by various bile salts as another possible index of the efficiency of bile salt absorption.

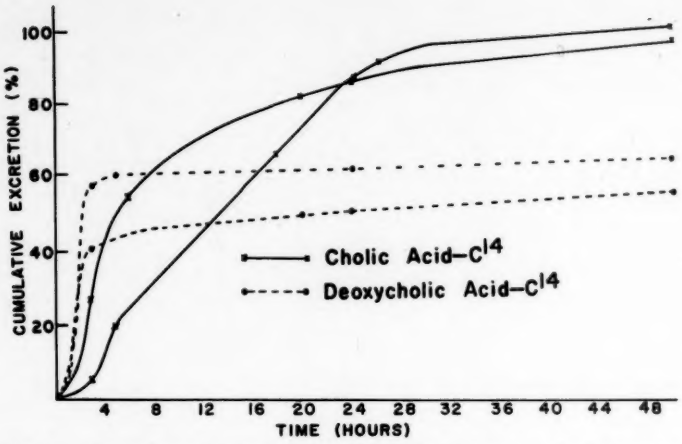

Fig. 3. Comparison of the twenty-four-hour biliary excretion of radioactivity after the intraduodenal administration of cholate-24-C^{14} and deoxycholate-24-C^{14}. From: PORTMAN, O. W. *Arch. Biochem.*, 78: 125, 1958.[11]

Cholesterol was fed at a 0.45 per cent level, and bile salts were fed at 0.15, 0.45 or 1.35 per cent levels. The bile salts that were evaluated were cholate, deoxycholate, 12-ketolithocholate, 7-ketodeoxycholate and dehydrocholate. There was a straight line serum cholesterol response with the logarithm of dietary cholate concentration but increasing dietary concentration of the other bile salts, which are bacterial metabolites of cholate, beyond the 0.15 per cent level did not result in increased serum cholesterol concentrations (Fig. 4). Although this experiment does not specifically demonstrate the absorption of one bile salt to be better than another, it is indirect evidence that there is a better absorption of cholate than of any of its bacterial metabolites which were tested.

We are unable to show conclusively that dietary influences on bile acid excretion are mediated through a more rapid degradation of biliary bile acids by the intestinal flora. In fact, little information is available about the specific microorganisms involved. It is natural to suspect that gram-negative anaerobes or facultative anaerobes are active. Bile salts strongly inhibit the growth of gram-positive organisms; this fact probably accounts for the relative scarcity of these organisms in the large intestine just as the even greater concentrations of bile acids in the small intestine probably contribute to the essential sterility of that portion of the intestine. Various workers have made some progress in studying the *in vitro* modification of bile acids by microorganisms.

TABLE VI
The Effect of the Type of Diet on the Serum Cholesterol Concentration of Rhesus Monkeys

Diet	Males (mg. %)	Females (mg. %)	Total (mg. %)
High sucrose.....	142.8*	152.0	147.4†
Chow-fruit.....	91.2	142.8	117.0

* N = Five per group.
† N = Ten per group.

EFFECTS ON SERUM CHOLESTEROL LEVELS

The observation that dietary factors influence the excretion of bile acids also suggested a possible influence of these factors on the concentrations of cholesterol in the blood and other tissues. It has been a rather common observation, although as far as I know one unemphasized in any publication, that the serum cholesterol level in rats fed a cholesterol-free diet is lower in rats fed Purina Laboratory Chow than that in rats fed apparently adequate purified diets. Mosbach et al.[13] have recently shown that the synthesis of cholesterol by livers of rats fed Purina Chow is much greater

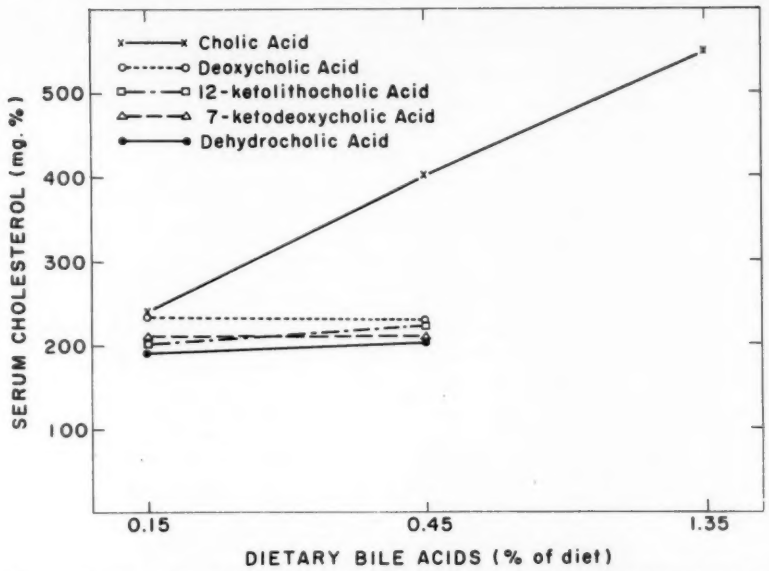

Fig. 4. A comparison of the serum cholesterol response of rats fed diets including 0.45 per cent cholesterol and 0.15, 0.45, and 1.35 per cent of each of the following bile acids: cholic, deoxycholic, 7-ketodeoxycholic,[19] 12-ketolithocholic[20] and dehydrocholic. Each point on the graph represents the mean of determinations at two weeks and four weeks for six rats.

than that of rats fed purified diets with sucrose. Also, as we reported previously,[14] the elevations in the levels of serum cholesterol and low density beta-lipoproteins induced by the feeding of cholesterol and cholic acid are less when cornstarch is the source of carbohydrate rather than glucose, fructose or sucrose. Differences of that type have been seen in most but not all experiments in which the type of carbohydrate was varied. Similar experiments performed on Cebus monkeys showed that the serum cholesterol level was only slightly lower in monkeys on diets containing cornstarch than in those on diets containing sucrose. Table VI shows the serum cholesterol values for a group of rhesus monkeys, half of which were fed a commercial laboratory diet plus fruits and half of which were fed a completely purified diet containing 18 per cent casein, 74 per cent sucrose and 4 per cent corn oil.* If both sexes of monkeys are included together, or if only the males are considered, there is a significant difference in the serum cholesterol levels of the different dietary groups.

Other researchers have also shown that the type of carbohydrate fed can influence the serum cholesterol level in rats[21,22] as well as in rabbits and chickens.[15] There are preliminary reports which indicate that the type of dietary carbohydrate may influence the serum cholesterol level in man.[23,24]

In our earlier studies, we demonstrated that partial sterilization of the large intestine by feeding Sulfasuxidine eliminated the hypocholesteremic effect of starch (compared to sucrose) in rats. Grant and Fahrenbach[15] in their studies with chicks have shown that the administration of chlortetracycline partially eliminates the carbohydrate effect. Danielsson and Gustafsson,[16] who previously demonstrated a decrease in bile acid excretion in the germ-free rat, have recently shown that serum cholesterol level in the germ-free rat is higher than in the normal or infected rat. Wells and Anderson[17] have shown that the feeding of lactose diets, which depress the cecal flora,

* I am indebted to Drs. Aina Auscaps and James Shaw of the Harvard School of Dental Medicine for supplying me with blood from these animals.

result in high serum cholesterol levels in rabbits. Because of these observations, it appears possible that the effect of varying the type of carbohydrate on the serum cholesterol level is mediated through effects on (1) the composition of the flora of the large intestine or (2) the gastrointestinal motility which in turn might vary the rate at which bile acids and neutral sterols are brought into the area of the intestinal microflora. Another possibility is that the greater residue in the cecum of the animals fed complex carbohydrate could reduce the efficiency of steroid reabsorption.

SUMMARY

A number of experiments which indicated that dietary factors can influence the biliary and fecal excretion of bile acids are presented. These studies indicate that the effects observed may be related to variations in the way in which bile acids are chemically changed in the cecum by intestinal microorganisms and to the rate at which unmodified and modified bile acids are reabsorbed. Rats fed diets which tend to promote excretion of greater quantities of bile acids appear also to have lower serum cholesterol levels.

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The Effect of Nicotinic Acid on Serum Lipids

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IN RECENT years there has been great interest in devising a practical clinical program to reduce elevated levels of serum cholesterol and especially of the low density beta-lipoproteins, because of their statistical relationship to atherosclerosis. There is no definite evidence that such reduction will result in retardation of atherogenesis or in removal of existing atheromas in human beings. However, any method of reducing serum cholesterol levels should be studied to determine its efficacy, safety and ultimate effect on the arteries. Finally, since hypercholesterolemia is a fairly common metabolic defect, reduction should be effected by chemotherapy rather than by any program which necessitates major alterations in diet. The distinction just made between dietary and non-dietary treatment is made for purposes of clinical investigation and does not imply that the two methods are considered mutually exclusive. However, since a dietary program has not yet been devised which will successfully reduce hypercholesterolemia in moderately severe or severe cases and still be acceptable to the majority of patients for an indefinite period of time, it seems likely that chemotherapy will represent the major if not the sole factor in the ultimate program for hypercholesterolemia.

STUDIES OF NICOTINIC ACID ADMINISTRATION IN HYPERCHOLESTEROLEMIA

The use of nicotinic acid in large doses for hypercholesterolemia was first suggested in 1955 by Altschul, Hoffer and Stephen,[1] who reported twenty-four–hour observations in eleven medical students and fifty-seven patients with hypercholesterolemia. The medical students received 4 gm. of nicotinic acid in divided doses and the patients received a single dose of 1 gm. Serum cholesterol levels were determined before the experiment and at the end of twenty-four hours. Those with control levels greater than 250 mg. per 100 ml. showed an average fall of 22 per cent in serum cholesterol; those with control levels less than 250 mg. per 100 ml. showed a fall of only 6 per cent. Nicotinamide, administered in a similar manner to twenty medical students, caused no effect on cholesterol levels. The report also stated that orally administered nicotinic acid had reduced cholesterol levels in twenty of twenty-four rabbits and that subcutaneous injection of nicotinic acid had produced a similar effect with a maximum reduction in four hours.[2]

In fall 1955 I initiated the first long term study in human subjects with Drs. Achor, Berge, McKenzie and Barker at the Mayo Clinic. Patients with hypercholesterolemia continuing their usual diets were given 3 gm. of nicotinic acid daily in divided doses after meals. This resulted in significant reduction of serum cholesterol levels in the majority. The reduction was most prominent in the beta-lipoprotein-cholesterol fraction and was sustained as long as the drug was administered. Total lipids were decreased; phospholipids were essentially unchanged.[3] (After the pilot study which was reported in June 1956, total lipids and phospholipids were no longer determined. Later work by others showed the phospholipid fraction to be reduced to some extent but to a lesser degree than cholesterol and total lipids.)

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TABLE I
Dosage Schedule

No. of Weeks	Dosage Given
0–12.....	3 gm. nicotinic acid daily (in divided doses after meals)
12–30.....	Same dose if cholesterol normal; if above 250 mg. per 100 ml. increased (4.5 gm., 6 gm., 7.5 gm. daily) at 6- to 8-week intervals
30–42.....	Nicotinamide in equal dosage
42–56.....	Resume nicotinic acid and adjust dose further if necessary (9 gm. in few)
56–60.....	No treatment
60 weeks on..	Resume nicotinic acid

TABLE II
Average Serum Cholesterol Levels (mg. per 100 ml.) Following Treatment with Nicotinic Acid and Nicotinamide

Group and No. of Patients	Serum Cholesterol Level				
	Pre-treatment	0–12 Weeks (nicotinic acid)	12–30 Weeks (nicotinic acid)	30–42 Weeks (nicotinamide)	42–56 Weeks (nicotinic acid)
1, 26	325	263	259	331	277
2, 16	312	269	260	336	244

METHODS OF STUDY

Since April 1956, I have conducted a similar long term study in which ninety patients have been treated and fifty are currently receiving nicotinic acid; twenty-nine patients have been in the study for more than two years and nineteen others have completed more than one year. The study is similar in design to that at the Mayo Clinic except that we use nicotinamide instead of a placebo for three months after the patients have received nicotinic acid for the first six months. Other details of material and methods have been reported previously.[4] Our schedule for treatment is outlined in Table I, a review of which will make it possible to understand the significance of other tables and graphs illustrating our results.

For the first twelve weeks each patient receives 3 gm. of nicotinic acid daily in divided doses after meals to minimize side reactions. If serum cholesterol and beta-lipoprotein cholesterol levels are normal at the end of this period, the same dose is continued. If the serum cholesterol is above 250 mg. per 100 ml. or the beta-lipoprotein cholesterol above 200 mg. per 100 ml., the daily dosage is increased to 4.5 gm. Later it may be increased to 6 gm. or 7.5 gm. at eight-week intervals, depending on the response. For a twelve-week period beginning after thirty or thirty-two weeks nicotinamide is substituted in equal dosage. Nicotinic acid administration is then resumed in the optimal dose and continued indefinitely with the exception of a four-week control period early in the second year of treatment.

TABLE III
Average Beta- and Alpha$_1$-Lipoprotein Cholesterol Levels (mg. per 100 ml.) Following Treatment with Nicotinic Acid and Nicotinamide*

Lipid Fraction	Lipoprotein Cholesterol Level				
	Pre-treatment	0–12 Weeks (nicotinic acid)	12–30 Weeks (nicotinic acid)	30–42 Weeks (nicotinamide)	42–56 Weeks (nicotinic acid)
Beta	202	153	152	224	147
Alpha	39	56	59	47	55
Beta: alpha$_1$	6.0	2.9	2.9	5.6	3.1

* Group 2 patients.

RESULTS

Table II shows the averages of all serum cholesterol levels in each of the periods of treatment in forty-two patients. The levels were reduced in the first twelve weeks of treatment and a slight further reduction in the averages occurred in the next eighteen weeks in which increased doses of nicotinic acid were given to more than half of the patients. Substitution of nicotinamide resulted in return of serum cholesterol levels to the pretreatment range. Resumption of therapy with nicotinic acid resulted in reduction in levels similar to the initial response.

Determination of lipoprotein cholesterol fractions (by electrophoresis and microchemical

TABLE IV

Serum Lipoprotein Cholesterol Fractions Following Nicotinic Acid Therapy: Comparison of Optimal Dosage with No Treatment in Thirty-Six Patients

Determination	No Treatment	Optimal Dose
Cholesterol (mg. per 100 ml.)	333	260
Beta-lipoprotein cholesterol (mg. per 100 ml.)	223	156
Alpha-lipoprotein cholesterol (mg. per 100 ml.)	55	63
Beta:alpha$_1$ ratio	4.7	2.8

determination of cholesterol) showed a considerable reduction in the beta-lipoprotein cholesterol fraction during nicotinic acid therapy (Table III). This was accompanied by a smaller but definite rise in the alpha$_1$ fraction. The beta:alpha$_1$ ratio was reduced to normal levels by treatment. Once again it can be seen that nicotinamide fails to duplicate the action of nicotinic acid on serum cholesterol levels and the lipoprotein fractions.

Table IV shows the magnitude of these changes by comparing the averages of all tests during optimal dosage of nicotinic acid in each patient with the periods in which the patients received nicotinamide or no treatment.

Figure 1 illustrates the response in one patient to several changes in dosage. The pretreatment value represents the average of several determinations. In the first sixteen weeks most serum cholesterol values were within the normal range while the patient received 3 gm. of nicotinic acid daily. When the dose was reduced to 1.5 gm. per day, three of the next four values were 300 mg. per 100 ml. or higher. On doses of 3 or 4.5 gm. the cholesterol levels returned to normal. When nicotinamide was substituted in a dose of 3 gm. daily for twelve weeks, the pretreatment average was duplicated. When nicotinic acid was again administered, levels within the normal range were once again attained.

Figure 2 shows a modest reduction below the pretreatment average when 3 gm. of nicotinic acid was administered daily. When the dose was increased to 4.5 gm., all values were between 150 and 250 mg. per 100 ml.

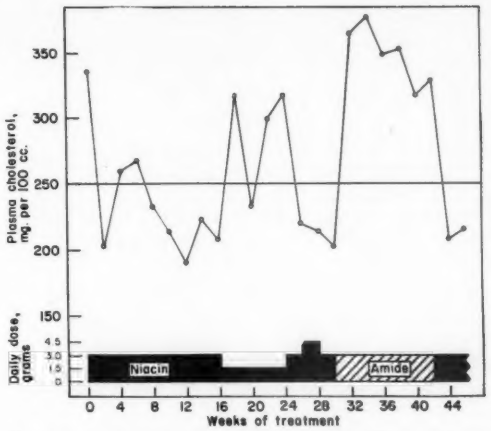

FIG. 1. Reduction of blood cholesterol levels by administration of 3 gm. of nicotinic acid (niacin) daily, return to elevated levels during use of 1.5 gm. of nicotinic acid daily, reduction to normal levels when a daily dose of 3 gm. of nicotinic acid was resumed, return to elevated levels when nicotinamide (niacinamide) was substituted, and reduction to normal levels when use of nicotinic acid was again resumed. (Figures 1 and 2 from: PARSONS, W. B., JR. and FLINN, J. H. Reduction in elevated blood cholesterol levels by large doses of nicotinic acid; preliminary report. *J.A.M.A.*, 165:234, 1917.)

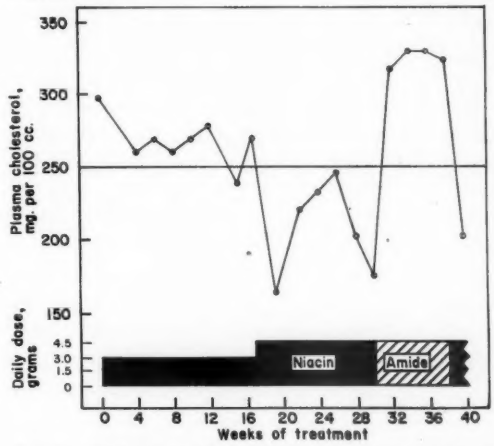

FIG. 2. Reduction of blood cholesterol levels to normal by administration of 4.5 gm. of nicotinic acid (niacin) daily, return to elevated levels when nicotinamide (niacinamide) was substituted, and reduction to normal levels when use of nicotinic acid was resumed.

Substitution of nicotinamide in equal dosage resulted in four consecutive values above 300 mg. per 100 ml. When the patient learned of this elevation, he insisted on returning to the

original medication, after which the serum cholesterol levels were again within the normal range.

COMMENTS

The effect of nicotinic acid on serum cholesterol levels has been confirmed by all investigators who have studied this method of treatment. Both free and esterified cholesterol fractions are reduced.[5] Those who have studied total lipids have found them to be reduced in the same manner as serum cholesterol.[5–10] Several reports indicate that phospholipids are reduced proportionately less than other fractions.[5, 6] Fatty acids and neutral fats are reduced,[7, 9] as are triglycerides (by calculation).[5] The observed changes in lipid fractions are identical with those produced by large doses of estrogens[11] or by administration of adequate amounts of unsaturated fat in place of saturated fat.[9]

Several miscellaneous observations deserve brief comment. The group at the Mayo Clinic has stated that with adequate amounts of nicotinic acid, at least 75 per cent of patients with hypercholesterolemia can maintain normal concentrations of blood lipids.[12] We would agree in general with this statement although the figure may be slightly higher than recent experience would indicate. We have been impressed by the number of patients in whom cholesterol levels have remained somewhat above normal and yet the beta-lipoprotein cholesterol fraction was consistently normal during treatment.[4] Other workers state that higher pretreatment levels are usually associated with greater reduction in serum cholesterol and that increased doses of nicotinic acid are usually associated with an increased response.[6] Our data confirmed the greater fall from higher levels, although the effect is not constant in patients and thus does not permit individual prediction. The relation of response to dosage is true within certain limits, but further reduction does not always occur if the initial dosage has resulted in normal levels, and there are refractory patients in whom no dosage we have yet employed (up to 9 or 10 gm. daily) results in reduction to normal or nearly normal cholesterol levels. Our experience does not support the statement that women respond better than men.[4, 6]

Gurian and Adlersberg[5] reported that the maximal effect of nicotinic acid administration on lipid levels takes several weeks to be realized and that the return to pretreatment levels after treatment is terminated also requires two weeks or longer. Although we have not studied these intervals in detail, our experience agrees with these observations. Altschul and Hoffer[2] reported a slight increase in basal metabolic rate in patients during treatment but thought that it was not enough to postulate a thyromimetic or thyrotropic effect. We agree with their conclusions.

Berge and co-workers[13] reported that sitosterol therapy has an additive effect when administered in conjunction with nicotinic acid. No effect was found when safflower oil was added without dietary restriction. Belle and Halpern[14] reported that patients previously treated with a low fat diet showed further significant reduction in cholesterol levels during nicotinic acid therapy. It can probably be assumed that methods of therapy which, when used together have an additive effect, must act in different ways or at different sites in cholesterol metabolism.

Effect on Tissue Cholesterol

An important consideration in any program of cholesterol reduction is the effect of the program on tissue cholesterol levels. Altschul[15] treated seventeen rabbits with large doses of nicotinic acid for ninety days while feeding them high cholesterol diets known from previous experience to produce severe atherosclerotic changes in the aortas in 90 per cent of the animals. He found that aortic lesions were entirely prevented in nine rabbits, mitigated in six and not influenced in the other two. Merrill and Lemley-Stone[16] found that the great rise in serum cholesterol usually seen in cholesterol-fed rabbits was partially prevented by administration of nicotinic acid, the final value being less than half that found in animals fed cholesterol but not given nicotinic acid. The average amount of cholesterol in the aortas of rabbits fed cholesterol without nicotinic acid was almost three times as much and

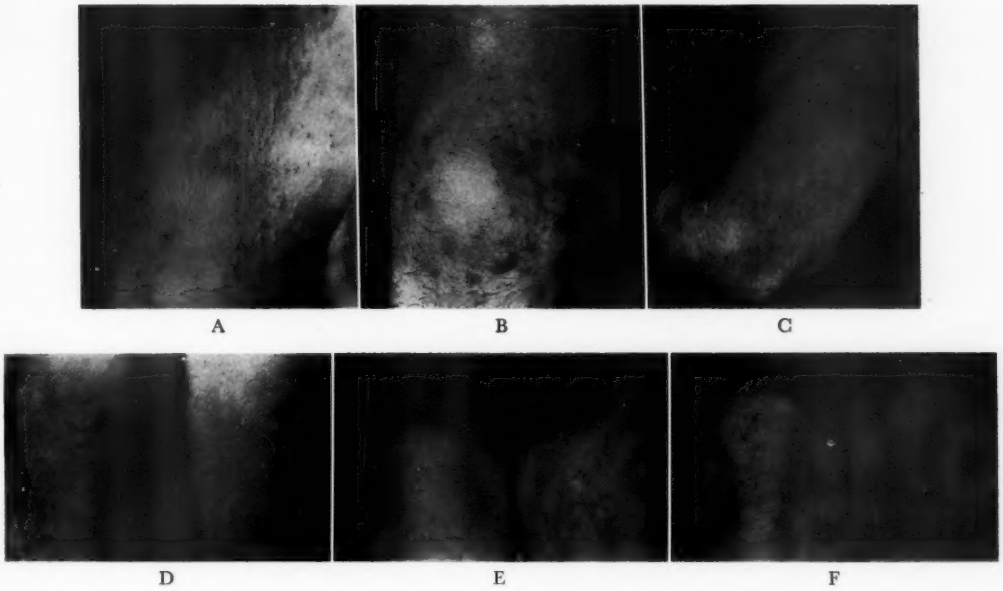

FIG. 3. Disappearance of xanthoma tuberosum in white man, age fifty-one, with arteriosclerosis obliterans. Nicotinic acid dosage, 3 gm. daily. A, right elbow before treatment; serum cholesterol level 736 mg. per 100 ml., beta-lipoprotein cholesterol (BLC) 540 mg. per 100 ml. B, right elbow after five months of treatment; serum cholesterol level 352 mg. per 100 ml., beta-lipoprotein cholesterol 216 mg. per 100 ml. Lesions much smaller and softer. C, right elbow after ten months of treatment; serum cholesterol level 152 mg. per 100 ml., beta-lipoprotein cholesterol 63 mg. per 100 ml. Lesions absent. D, knees before treatment. E, knees after five months of treatment. Lesions smaller and softer. F, knees after ten months of treatment. Lesions barely perceptible on right knee, absent from left knee.

the average amount of liver cholesterol more than four times as much as those who received the drug. On the basis of these experiments it must be concluded that nicotinic acid has a potent influence on cholesterol metabolism in rabbits, resulting in considerable protection against cholesterol deposition in the aorta and the liver. As always, caution must be exercised in applying the results of experiments on animals to man. Insufficient time has elapsed to determine the possible influence of nicotinic acid on atherosclerosis in human patients, comparing large numbers of treated patients with a control group.

Disappearance of cholesterol deposits in the skin has been observed during nicotinic acid therapy. Xanthelasmas have disappeared from the eyelids of patients treated by other investigators.[11, 17] The changes occur slowly over a period of several months, sometimes requiring a year or longer. Xanthelasmas have not disappeared in any of our patients. On the other hand, we have observed a striking demonstration of disappearance of xanthoma tuberosum in the only patient with this lesion followed up in the study for any length of time. His lesions of the skin disappeared completely after ten months of treatment. This change was associated with a reduction in serum cholesterol and beta-lipoprotein cholesterol levels from high levels to values in the low normal range. These changes are illustrated in Figure 3.

Recently a second patient with xanthoma tuberosum and high lipid values entered the study. His serum lipids have been strikingly reduced in the first four weeks of treatment, but a longer time is necessary to assess the effect on his xanthomas. To our knowledge reduction in size of the lesions of xanthoma tendinosum has not been reported.

A significant number of patients (45 per cent

in our latest tabulation) stated that they experience dryness of the skin while taking nicotinic acid. We suspect that this represents reduction in the cholesterol content of the skin, but this has not yet been confirmed by appropriate studies.

Side Effects of Nicotinic Acid Therapy

Side reactions to nicotinic acid should be mentioned. The cutaneous flush and pruritus which occur following ingestion of nicotinic acid tend to disappear rapidly in the first week of treatment; by the end of the second week they have disappeared in at least two-thirds of patients. About one-third of the patients state that the flush remains longer than two weeks and 10 per cent state that flush continues to occur after every dose although it is mild and does not interfere with treatment. About half of our patients who had flush beyond two weeks stated that they had sufficient discomfort to desire a preparation which would eliminate it. Attempts to produce such a preparation have thus far met with only moderate success, but we have not been concerned about the flush since it has not represented a problem in the conduct of the study.

Gastrointestinal irritation has been a more serious problem. Although immediate nausea and vomiting occur in few patients, the most distressing effects may appear after one or two years of continuous therapy, at which time peptic ulcers have been activated in five patients. This effect is undoubtedly related to the acidity of plain nicotinic acid preparations, since substitution of a buffered preparation results in relief of symptoms and has permitted these patients to continue treatment.

Further Metabolic Effects ("Toxic" Effects) of Nicotinic Acid

The apparent safety of nicotinic acid has been mentioned by most investigators, but almost without exception the published statements have been based on observations covering periods of one year or less. Our first two years of experience failed to show any significant alterations in hematologic studies, urinalyses, blood glucose or non-protein nitrogen levels, or a battery of tests of liver function. Needle biopsies of the liver in seventeen patients after more than one year of treatment were reviewed by two pathologists and showed no significant abnormalities.[4] However, in our third year of study, possible evidence of hepatic dysfunction has led us to reconsider the question of hepatotoxicity.

Most abnormalities have appeared in the bromsulfalein test, which has been abnormal on two consecutive determinations in eight patients. Two other patients showed slight retention on a single test but had a normal response when the test was repeated without discontinuing therapy. Of the eight patients showing repeated abnormalities in retention of dye, four have thus far had needle biopsies, the results of which have varied sufficiently to make their significance uncertain. One biopsy specimen showed normal liver tissue. Another showed marked fatty metamorphosis, but this was from a patient newly discovered whose diabetes was as yet uncontrolled at the time of the biopsy. The fourth patient showed changes consistent with cholangiolitis but not the picture usually found in jaundice due to chlorpromazine or methyltestosterone. Recently a single well documented case report was published[18] which suggests the occurrence of intrahepatic obstructive jaundice following the use of nicotinic acid. We have two patients in whom the possibility of intrahepatic cholestasis must be considered. Detailed reports are beyond the scope of this presentation but will be published elsewhere.

Gurian and Adlersberg[5] reported marked reduction of tolerance to glucose including the appearance of frank diabetes as evidenced by elevated fasting blood sugar levels as well as diabetic glucose tolerance curves in patients taking large doses of nicotinic acid. These changes reverted to normal when administration of the drug was discontinued. We have found that nicotinic acid does not impair the control of adult-onset diabetes, an observation also made by others. Within the past year, diabetes has developed in three of our patients. All three were middle-aged men who were somewhat overweight; the father of one had diabetes. All three of these men have also showed marked changes in tests of liver

function, either before or after discovery of diabetes. Whether the diabetes and dysfunction of the liver are coincidentally or significantly associated remains to be seen. Two of the patients continued to take nicotinic acid during regulation of diabetes by weight reduction, using a diabetic diet. In the other patient administration of the drug was stopped because of dysfunction of the liver but was resumed three months later without any untoward effect on the diabetes which had been controlled by weight reduction and diet.

Twenty-two of thirty patients tested after one to three years of treatment had serum uric acid levels in excess of our normal range of 3 to 6 mg. per 100 ml. We had not performed pretreatment uric acid tests on any of these patients. Subsequently we tested twelve patients before and during treatment. Another thirteen were tested during treatment and following a control period. The average serum uric acid levels for both groups were significantly higher during treatment. Uric acid excretion in twenty-four–hour urine specimens from these patients have shown that they excrete normal amounts of uric acid. No instances of gout or renal calculi have been observed. The significance of these observations is unknown.

Mechanism of Hypocholesterolemic Action of Nicotinic Acid

The mechanism of action by which nicotinic acid reduces blood lipid levels is still unknown. The opinion has frequently been expressed that nicotinic acid might cause depletion of methyl groups in the liver. However, the failure of nicotinamide to produce changes in serum lipids is against such a view. Furthermore, Miller and associates[10] found that administration of methionine in conjunction with nicotinic acid failed to modify the effect of the latter on serum lipid levels. They observed that excretion of methylated metabolites of nicotinic acid is greater when nicotinamide is administered and yet nicotinamide has no effect on serum lipids. The failure of choline and methionine to alter the effect of nicotinic acid in rats and in man was also observed by Olson et al.[19]

The possibility of a stress effect mediated through the adrenals has been suggested in view of the flush which accompanies nicotinic acid therapy. We have considered from the start that this was impossible in view of the maintenance of normal cholesterol levels long after the flush has disappeared in almost all patients. Furthermore, we have numerous patients in whom an increase in dosage of nicotinic acid has resulted in reduction of cholesterol despite the fact that no flush occurred with the larger dose. To our knowledge no studies have been carried out to refute the adrenal stress theory, but we believe it is the obligation of its proponents to furnish evidence to support it.

In a study reported a year ago, Miller, Hamilton and Goldsmith[10] found a significant difference in products of urinary excretion during therapy with nicotinic acid and with nicotinamide. The main difference was that about 20 to 25 per cent of nicotinic acid was excreted as nicotinuric acid, whereas none of this metabolite was excreted during nicotinamide therapy. The excretion of n-methyl nicotinamide was considerably higher (about twice as much) during nicotinamide treatment. These observations suggested that nicotinuric acid, an intermediary metabolite of nicotinic acid, could play a role in the metabolism of cholesterol. We have not had a sufficient supply of nicotinuric acid to perform a full-scale study of its effect, but our limited experience indicates that it is probably not active in reducing cholesterol levels in doses of 0.5 to 3 gm. per day.

We have recently begun studies of incorporation of carbon^{14}-labeled acetate into cholesterol in patients being treated with nicotinic acid and in the same patients while they are receiving no treatment or nicotinamide but no results can yet be reported. It will be interesting to see whether our results in human patients are similar to any of the studies performed on animals thus far using C^{14}-labeled acetate. Merrill[20] fed nicotinic acid to rats and observed increased incorporation of sodium acetate into cholesterol. Similarly, nicotinic acid added to rat liver slices *in vitro* increased the incorporation of radiocarbon into cholesterol. How-

ever, Duncan and Best[21] did not observe any influence of nicotinic acid on incorporation of C^{14} in liver and serum cholesterol after intraperitoneal injection of labeled acetate. They also found no interference with gastrointestinal absorption of cholesterol or evidence of increased fecal excretion. Oxygen consumption and thyroid weights were determined to see whether or not nicotinic acid had any effect on thyroid function, but no significant change was found. Perry[22] has recently reported that in rat's liver slices nicotinic acid therapy results in considerably larger amounts of labeled acetate being metabolized directly to carbon dioxide rather than converted into cholesterol.

There appears to be increasing evidence that nicotinic acid (or perhaps a metabolite) reversibly alters an enzyme system in the liver which participates in synthesis of cholesterol. If this system should prove to be related to glycine conjugation (which is important in excretion of bromsulfalein), it would explain the observation of rapidly reversible dysfunction of the liver as indicated by retention of bromsulfalein without pathologic evidence of hepatocellular damage. It could also explain the failure of nicotinamide to alter the lipid pattern of the blood in contrast to the effect of nicotinic acid, except that other compounds which are glycine acceptors are ineffective in changing serum cholesterol levels.[10]

CONCLUSIONS

Until there is evidence of a favorable effect upon atherosclerotic lesions, not only nicotinic acid but every other agent currently known to reduce serum cholesterol levels must be considered experimental. For this reason no program to lower cholesterol levels should be undertaken unless both the physician and the patient understand that they are participating in an experiment. Most investigators who have studied this agent believe that nicotinic acid is probably the most effective agent introduced to date for reducing serum levels of cholesterol and beta-lipoprotein cholesterol. However, the possibilities of dysfunction of the liver, decreased carbohydrate tolerance and increased serum uric acid levels after prolonged therapy dictate caution in its use until the full significance of these changes is known. If nicotinic acid itself should prove to have adverse effects which make its clinical use unwise, it is possible that an analogue or metabolite will be developed which will favorably alter serum lipids without toxicity. Further exploration of the differences between the action of nicotinic acid and its amide should lead to a more complete understanding of the mode of action and possibly of the regulatory mechanism for cholesterol and associated serum lipids as well.

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Investigation of the Mechanism of Action of Nicotinic Acid on Serum Lipid Levels in Man

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ALTSCHUL'S[1] observation that the administration of large doses of nicotinic acid to human subjects lowered serum cholesterol levels and that administration of an equal amount of nicotinamide was without effect has stimulated a great deal of interest. This observation has been confirmed by Parsons et al.[2] and others,[3,4] as well as by work carried out in our laboratory. A preliminary report of some of our results has been published.[5]

Administration of nicotinic acid may influence serum lipid concentrations by one or more mechanisms: (1) by increasing the excretion of sterols (cholesterol and coprosterol) in the feces and perhaps also excretion of bile acids; (2) by having some effect on the lipid transport mechanism in the blood; (3) by influencing the biosynthesis of lipids, particularly cholesterol; or (4) by having some action relative to the distribution of cholesterol and other lipids between the vascular and the cellular compartments.

Initially, the hypothesis that nicotinic acid had some effect on the excretion of lipids into the stool was entertained and studies were designed accordingly. It became evident that this was not the explanation for the action of nicotinic acid in lowering serum lipids, and other avenues of approach were considered.

The inactivity of nicotinamide in lowering serum cholesterol levels leads to speculation that the carboxyl group of nicotinic acid may be involved in the mechanism by which nicotinic acid produces a hypolipemic effect. This does not rule out the possibility that another reactive site on the molecule might also be implicated. For example, it is well known that the body methylates nicotinamide, to form N′-methylnicotinamide as well as N′-methylnicotinamide 6-pyridone, and that the major part of the nicotinic acid metabolites is excreted as these methylated products. It has been established that the methylation of nicotinic acid is dependent on the metabolism of lipotropic factors such as choline and methionine. It therefore seemed desirable to determine whether or not methyl metabolism is involved in the mechanism under investigation.

METHODS

All studies to be reported were conducted with selected patients who had moderate or marked hypercholesteremia. They received a diet of natural foods and were maintained on a metabolic ward. The diet of the first subject (G) contained about 40 gm. of fat, most of which was derived from animal products, 60 gm. of protein and sufficient calories to maintain body weight. The second subject (McS) received a diet which differed from that

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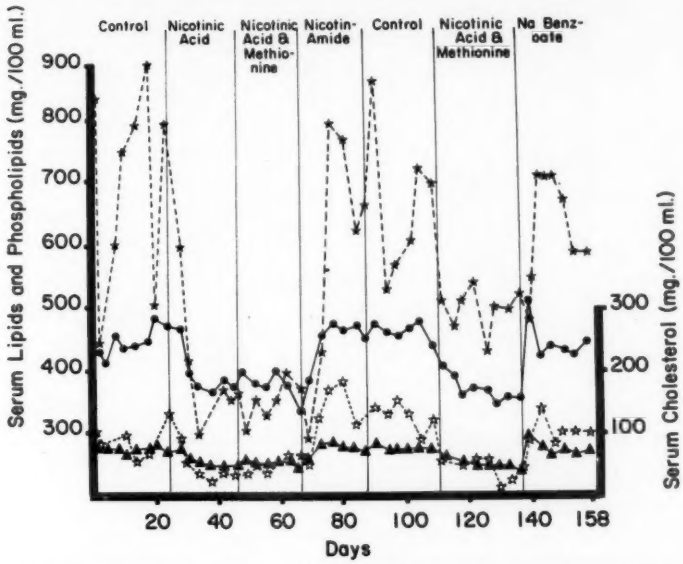

FIG. 1. The effect of administration of nicotinic acid, methionine, nicotinamide and sodium benzoate upon serum lipid levels (subject G). The closed circles connected by a solid line represent total cholesterol; closed triangles connected by a solid line represent free cholesterol; closed stars connected by a broken line represent total lipid esters; and open stars connected by a broken line represent phospholipids. All values are plotted against time in days.

of the first in that it furnished 115 gm. of animal fat. The caloric intake was adjusted by removing an isocaloric amount of carbohydrate. Collections of stools and urine were made continuously throughout the study. Blood was drawn twice weekly and the following determinations were carried out: (1) cholesterol by the method of Schoenheimer and Sperry;[6] (2) phospholipids as phospholipid phosphorous by the method of Horecker et al.,[7] and (3) total lipid esters by the method of Hack.[8]

Stools were analyzed for sterols and bile acids by methods developed in our laboratory. Sterols were determined by glass paper chromatography,[9] and bile acids by a procedure in which the bile acids were titrated with a standard base.[10] Urines were analyzed for N′-methylnicotinamide (N′-Me) N′-methylnicotinamide 6-pyridone (pyridone), nicotinic acid and nicotinuric acid.[11–14] In addition, tests of liver function and routine hematologic studies were carried out once weekly during the early part of these studies.

The control and experimental therapeutic periods lasted for three weeks unless otherwise indicated. Following an initial control period, 1 gm. of nicotinic acid was administered three times a day to the first subject (G). In the next period 2 gm. of DL-methionine plus 1 gm. of nicotinic acid were given three times daily. Subsequently, nicotinamide was substituted for nicotinic acid at the level of 1 gm. three times a day and methionine was withdrawn. This was followed by another control period. In the next period nicotinic acid plus DL-methionine was administered as described. In the final period an equimolar amount of sodium benzoate was substituted for nicotinic acid and methionine was withdrawn.

RESULTS

The data illustrated in Figure 1 demonstrate the effect of various types of therapy on the serum lipid levels of subject G. It can be seen that the administration of nicotinic acid resulted in the lowering of total cholesterol, free cholesterol, total lipid esters and phos-

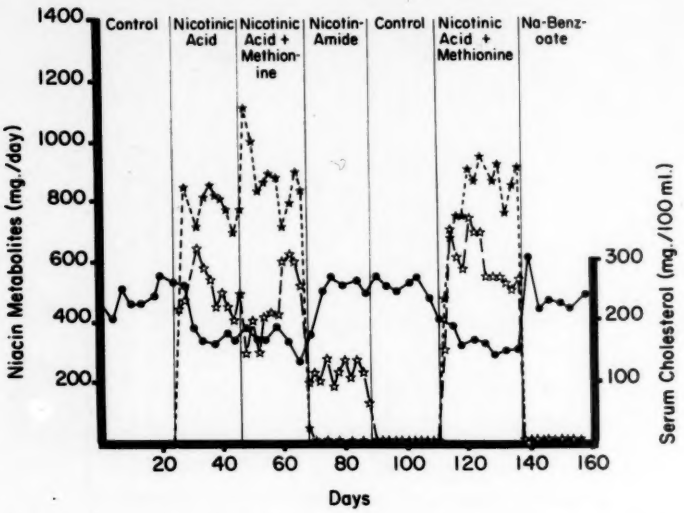

FIG. 2. The effect of administration of nicotinic acid, methionine, nicotinamide and sodium benzoate upon the urinary excretion of nicotinic acid and nicotinuric acid (subject G). Closed stars connected by a broken line represent nicotinuric acid excretion; open stars connected by a broken line represent nicotinic acid excretion. Other symbols as in Figure 1.

pholipids. In other words, all serum lipids were lowered. After the lipid levels had been lowered, methionine was added in order to supply methyl groups for methylation of nicotinic acid metabolites. No significant change in blood lipid levels was observed. This suggests that the effect of the administration of nicotinic acid is not the result of an increasing demand for labile-methyl donors and thus lowers the serum lipids by depleting the supply of lipotropic factors available for lipid transport. When nicotinamide was substituted for nicotinic acid and methionine, the serum lipid levels observed in the initial control period were re-established. It seemed possible that methionine might prevent the lowering of serum lipids by nicotinic acid administration even though it did not reverse the action of this compound. Therefore, nicotinic acid and methionine were given together following another control period. Levels of serum cholesterol, phospholipids and lipid esters dropped to the same extent as when nicotinic acid was given alone.

It occurred to us that urinary excretion of niacin metabolites might give an indication as to the active metabolite responsible for lowering serum cholesterol levels. It is known that the body conjugates glycine to nicotinic acid to form nicotinuric acid and that this compound is a major excretory product when large amounts of nicotinic acid are administered. The same type of condensation reaction occurs in the case of bile acids which are excreted in the bile as conjugated bile acids. Therefore, it appeared that sodium benzoate, which requires glycine for detoxification, might be effective in lowering serum cholesterol levels if the action of nicotinic acid was due to an increased demand for glycine production. The data in the last period demonstrate that sodium benzoate substituted for equimolar amounts of nicotinic acid was ineffective.

Urinary excretion of nicotinic acid and nicotinuric acid during each of the eight periods is shown in Figure 2. The total serum cholesterol level is plotted on the same figure for reference. During the control period excretion of nicotinic acid and nicotinuric acid in the urine was low. Of interest is the fact that in the periods in which nicotinic acid was given alone, nicotinuric acid composed a major por-

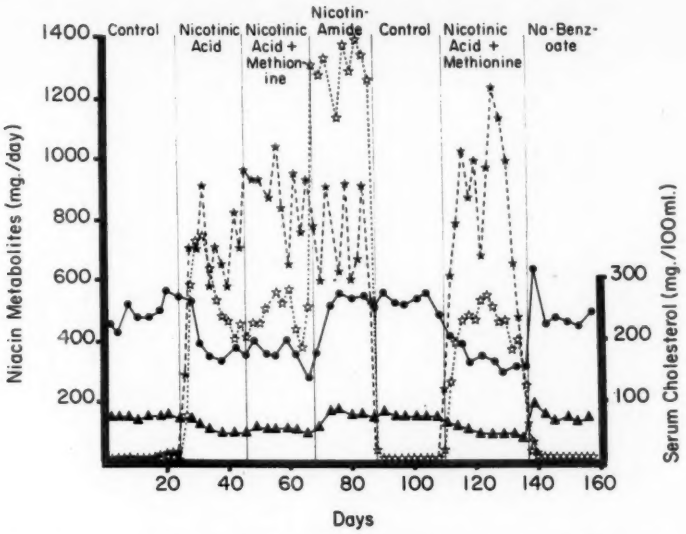

FIG. 3. The effect of administration of nicotinic acid, methionine, nicotinamide, and sodium benzoate upon the urinary excretion of N′-Me and pyridone (subject G). Closed stars connected by a broken line represent pyridone excretion; open stars connected by a broken line represent N′-Me excretion; and closed triangles represent serum free cholesterol. Other symbols as in Figure 1.

tion of the niacin metabolites excreted. The ratio between excretion of nicotinic acid and that of nicotinuric acid was not affected by the administration of methionine. However, when nicotinamide was given, nicotinic acid and/or nicotinamide was found in the urine but no nicotinuric acid was excreted. During the period in which nicotinic acid and methionine were given simultaneously it was observed that nicotinuric acid again was a major urinary metabolite. Only minimal amounts of nicotinic acid and nicotinuric acid were observed in the periods in which no therapy was given.

Figure 3 shows the urinary excretion of other niacin metabolites, N′-Me and pyridone. It is interesting that less N′-Me than pyridone was excreted during periods in which nicotinic acid was given, whereas the reverse was true when nicotinamide replaced nicotinic acid. The total methylated products which appeared in the urine increased during administration of nicotinamide because of a large increase in N′-Me excretion. These data would appear to eliminate both N′-Me and pyridone from a role in lowering serum lipid levels.

The hypothesis that administration of nicotinic acid might lower serum lipids because of its effect on the excretion of bile acids and sterols in the stools was tested in this and other experiments previously reported.[5,10] Figure 4 demonstrates that administration of nicotinic acid has no effect on bile acid or sterol excretion. Although fecal excretion of bile acids and sterols decreased after the first control period, it remained low during all experimental periods and in the second control period. This decrease was obviously not related to medication and presumably was due to the change from an average hospital diet, which was relatively high in fat, to the control diet which furnished only 40 gm. of fat. During the experimental periods, about 500 mg. of bile acids were excreted per day regardless of the therapeutic regimen. An additional 500 mg. of fecal sterols were excreted, making a total of about 1 gm. of steroids excreted per day. Although the sterols were determined so that cholesterol and beta-sitosterol were determined together, coprosterol was determined separately. In this study no

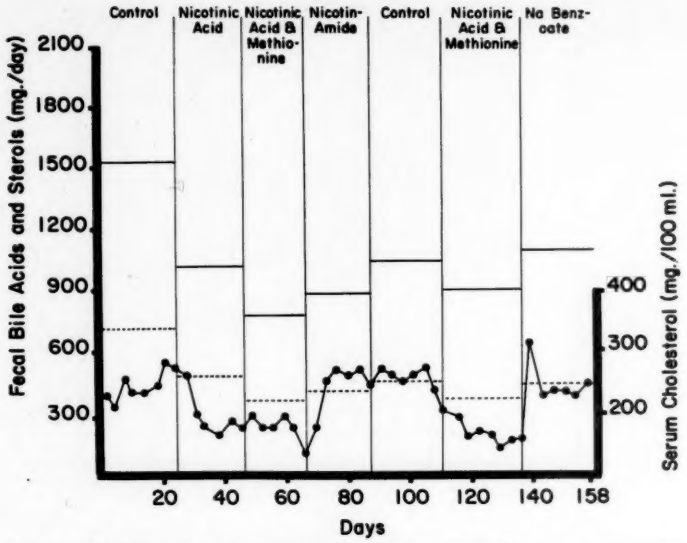

FIG. 4. The effect of administration of nicotinic acid, methionine, nicotinamide and sodium benzoate on fecal excretion of bile acids and sterols (subject G). Area under the solid line represents total bile acid and sterol excretion and the area under broken line represents sterol excretion. Other symbols as in Figure 1.

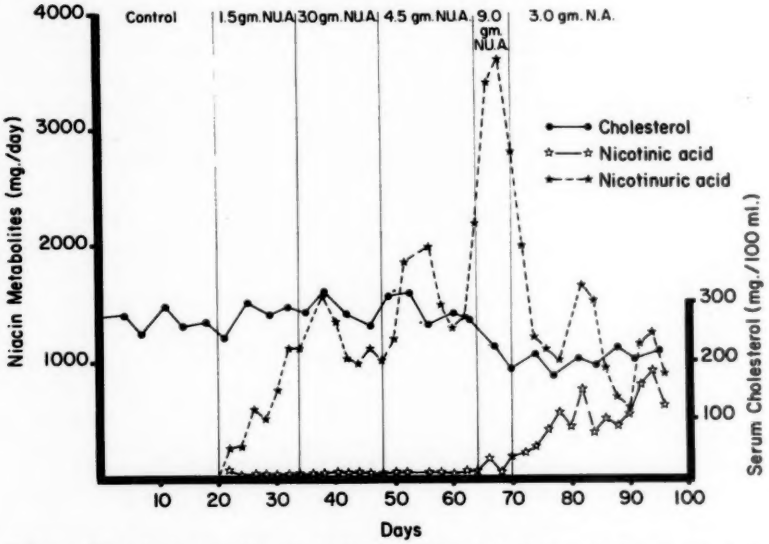

FIG. 5. The urinary excretion of nicotinuric acid and nicotinic acid after the administration of nicotinuric acid (NU.A.) and nicotinic acid (N.A.) is plotted against time in days (subject G).

significance could be attached to the ratio of coprosterol to the other sterols.

In view of the large amount of nicotinuric acid excreted in the urine during therapy with nicotinic acid and the complete absence of this metabolite when nicotinamide was given, we wanted to determine whether or not nicotinuric acid might be the form of nicotinic

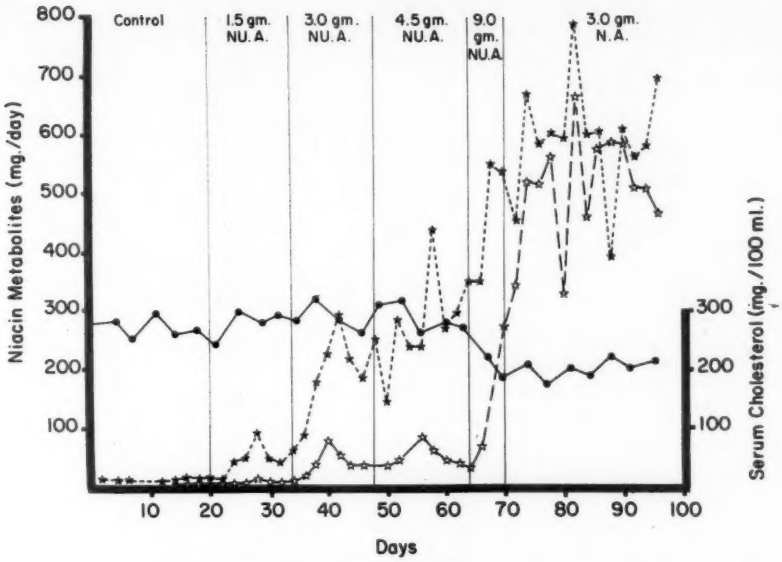

FIG. 6. The effect of administration of nicotinuric acid (NU.A.) and nicotinic acid (N.A.) on the urinary excretion of N′-Me and pyridone, (subject G). Closed stars connected by a broken line represent urinary excretion of pyridone, open stars connected by solid line represent excretion of N′-Me. Other symbols as in Figure 1.

acid responsible for lowering serum cholesterol. The same patient (G) was studied as in the preceding experiments (Figs. 1 to 4). A time lapse of several months necessitated a new control period to establish the average serum cholesterol level on the standard diet. At the end of twenty days, nicotinuric acid was given in the amount of 0.5 gm. three times a day and was continued for fourteen days (Fig. 5). Cholesterol levels were unchanged. Excretion of nicotinic acid in the urine remained at practically zero while there was a gradual increase in the excretion of nicotinuric acid. The dose of nicotinuric acid was then increased to 1 gm. three times daily and this therapy was continued for another two weeks. Excretion of nicotinuric acid remained about the same as in the previous period. During the next period the dose was increased to 4.5 gm. per day in three, equally divided doses. Urinary excretion of nicotinuric acid increased and still no free nicotinic acid appeared in the urine. After sixteen days at this dosage, the serum cholesterol level may have decreased slightly. Accordingly, the dose was increased to 3 gm. three times daily and continued for only six days as the supply of nicotinuric acid was exhausted. With this dosage, a large increase in the urinary excretion of nicotinuric acid was observed with only a minor increase in free nicotinic acid. After therapy with nicotinuric acid was discontinued the patient received 1 gm. of nicotinic acid three times a day to again test the response to nicotinic acid. The last serum cholesterol level determination taken before the end of the nicotinuric acid therapy was as low as the average level attained during nicotinic acid therapy. Therefore, it appears that nicotinuric acid had induced a lowering of serum cholesterol which was maintained without further decrease by nicotinic acid.

Comparing the urinary excretion of niacin metabolites (Figs. 5 and 6) during administration of nicotinuric acid at 4.5 gm. per day with the administration of equimolar amounts of nicotinic acid (3 gm. per day), 59 per cent of the dose of nicotinuric acid could be accounted for in the urine, as compared to 88 per cent of the dose of nicotinie acid. Even when nico-

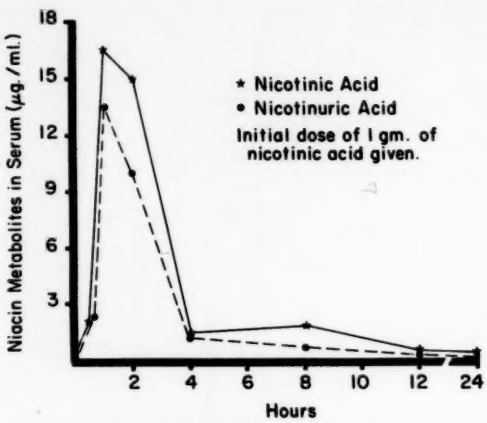

FIG. 7. Concentration of nicotinic acid and nicotinuric acid in serum after the administration of 1 gm. of nicotinic acid is plotted as a function of time (subject G).

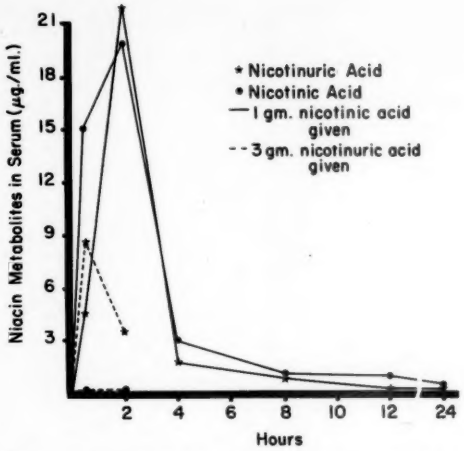

FIG. 8. Concentration of nicotinic acid and nicotinuric acid in serum after the oral administration of nicotinic acid and nicotinuric acid is plotted as a function of time (subject G). The dose of nicotinic acid was administered after three weeks of therapy with nicotinic acid (3 gm. per day). Nicotinuric acid was administered after the subject had responded to therapy with nicotinuric acid as indicated by a lowered serum cholesterol.

tinuric acid administration was doubled (9 gm. per day) only 59 per cent of the dose was found in the urine as niacin metabolites. These data suggest that nicotinuric acid was poorly absorbed. Little of the nicotinuric acid was converted to nicotinic acid when nicotinuric acid was administered (Fig. 5). However, data shown in Figure 6 demonstrate that an appreciable amount of nicotinuric acid was converted to nicotinic acid metabolites; in periods in which nicotinuric acid was given, increasing amounts of pyridone were excreted in the urine whereas relatively small amounts of the N′-Me were excreted. Comparing the urinary excretion of methylated niacin metabolites during administration of 4.5 gm. of nicotinuric acid per day with equimolar amounts of nicotinic acid (3 gm. per day), only 1.5 per cent of the dose of nicotinuric acid was excreted as N′-Me whereas 15 per cent of the dose of nicotinic acid was excreted as this metabolite (Fig. 6). Similarly, 8 per cent of the dose of nicotinuric acid was excreted in the urine as pyridone as compared to 16 per cent of the dose of nicotinic acid. These data suggest that the conversion of nicotinuric acid to nicotinamide, which is in turn methylated, is slow. If this were not true, a greater proportion of the methylated niacin metabolites presumably would be excreted in the form of N′-Me rather than as pyridone (Fig. 3).

Although the data presented in Figures 5 and 6 indicate that the conversion of nicotinuric acid to nicotinic acid is slow and that practically no nicotinic acid is excreted in the urine when nicotinuric acid is given orally, the possibility that appreciable amounts of nicotinic acid may be present in the blood at various times during the therapy is not ruled out. Nicotinic acid *per se* could still be the active agent rather than nicotinuric acid in lowering serum lipids.

Accordingly, investigations of blood levels of nicotinuric acid and nicotinic acid were initiated. The concentration of nicotinic acid in serum during fasting is less than 0.15 μg. per ml. in subjects who have not received nicotinic acid. The increase in serum levels observed when 1 gm. of nicotinic acid was given to a patient (subject G) who had received no previous therapy is shown in Figure 7. The concentration of nicotinic acid in serum rose 16.5 μg. per ml. within one hour. Nicotinuric acid in serum was 13.5 μg. per ml. at this time. Figure 8 shows the serum levels observed in a patient (subject G) who received 1 gm. of nicotinic acid after three weeks of therapy with 3 gm. of nicotinic acid daily. The maximum levels of nicotinuric acid and nicotinic

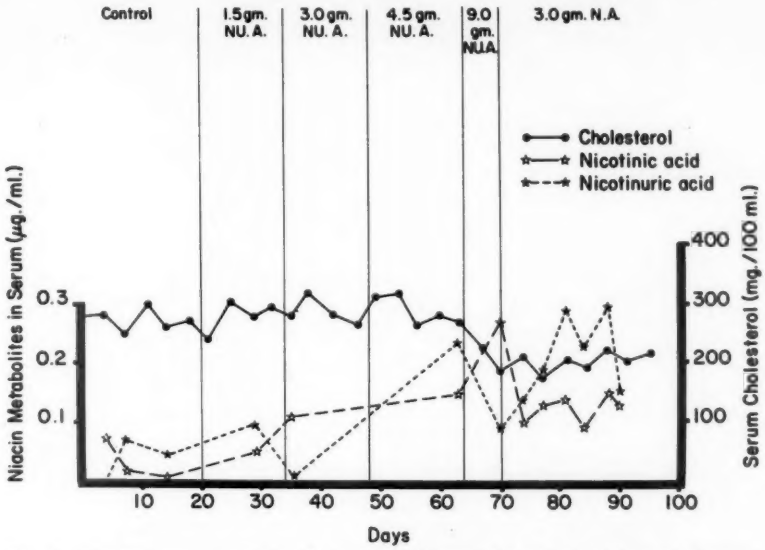

FIG. 9. Fasting serum concentrations of nicotinuric acid and nicotinic acid after the administration of nicotinuric acid (NU.A.) and nicotinic acid (N.A.) is plotted against time in days (subject G).

acid are approximately 50 per cent and 20 per cent higher, respectively, than after the first dose of nicotinic acid.

The same patient was given 3 gm. of nicotinuric acid at the end of the period in which 3 gm. of nicotinuric acid were given three times a day for six days (Fig. 8). A maximum value of 8.7 μg. per ml. of nicotinuric acid was obtained. This is only 40 per cent of the value obtained when 1 gm. of nicotinic acid was given, although the nicotinuric acid was given at a dose level twice the equivalent weight of nicotinic acid. It is apparent that absorption of nicotinuric acid is relatively poor. A significant finding is that very little, if any, nicotinic acid was detected in the blood. This study was performed at a time when the patient had responded to nicotinuric acid therapy as judged by a lowered serum cholesterol level. These data suggest that nicotinuric acid and not nicotinic acid is responsible for the decrease in the lipids in serum.

Figure 9 shows the fasting levels of nicotinic acid and nicotinuric acid in serum in patient G during the studies in which varying doses of nicotinuric acid were administered. Nicotinuric acid levels in serum were 0.10 to 0.15 μg. per ml. during the period of therapy with nicotinuric acid when there was no decrease in serum cholesterol. After therapy had been changed from 9 gm. of nicotinuric acid to 3 gm. of nicotinic acid per day, and when serum cholesterol concentration had decreased, nicotinic acid levels in serum ranged from 0.09 to 0.15 μg. per ml. These data support the view that the nicotinic acid level in the serum *per se* is not related to the effect of nicotinic acid in lowering cholesterol levels. It should be noted also that concentrations of nicotinuric acid appeared to be about twice as high as those of nicotinic acid after the patient had responded to nicotinic acid therapy with a decrease in serum cholesterol levels. These values for nicotinuric acid were generally higher than those found when nicotinuric acid was administered in amounts which had no effect on serum cholesterol levels.

Another patient (McS) was started on nicotinic acid therapy at the level of 1 gm. three times a day after a control period (Fig. 10). Administration of nicotinic acid lowered the serum cholesterol level. After twenty-one days, therapy was discontinued and another control period of twenty-two days was instituted. Therapy with nicotinuric acid at the level of 5 gm. three times a day was given for

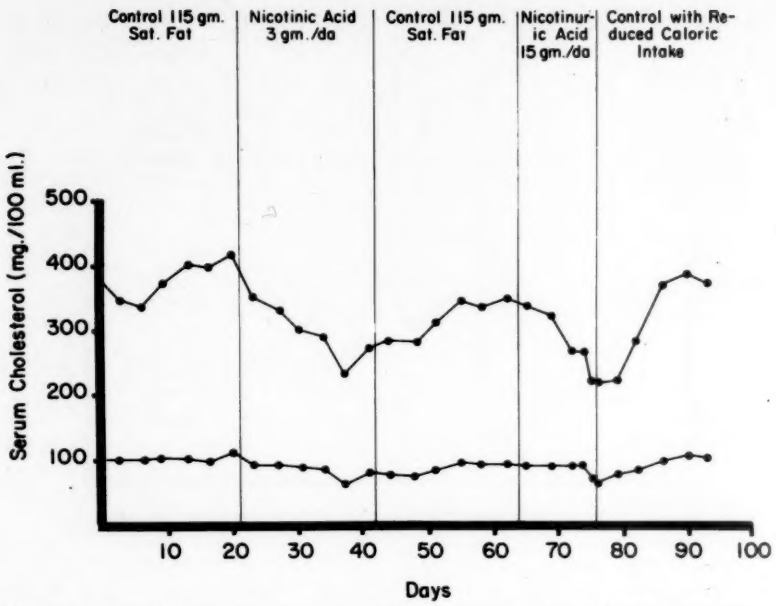

FIG. 10. Concentration of total cholesterol in serum (*top line*) and free cholesterol (*bottom line*) after the administration of nicotinic acid and nicotinuric acid is plotted against time in days (subject McS).

eleven days. This large dose was chosen in view of the apparently poor absorption of this compound. Blood levels of nicotinuric acid and nicotinic acid were determined after the first 5 gm. dose of nicotinuric acid (Fig. 11). The maximum level of nicotinuric acid was attained in four hours and was about 35 per cent higher than that resulting from a dose of 3 gm. of nicotinuric acid given to subject G after six days of therapy (Fig. 8). During the time in which the blood level of nicotinuric acid was high, only minimal amounts of nicotinic acid were found in the serum. This patient responded to nicotinuric acid therapy (Fig. 10), and the serum cholesterol level dropped precipitously. Serum concentrations of nicotinuric acid and nicotinic acid during fasting were determined daily during the last four days of the experimental period with nicotinuric acid. Even after eleven days of therapy with 15 gm. of nicotinuric acid daily, less than 5 per cent of the total nicotinic acid metabolites in the blood was present in the form of nicotinic acid, whereas 95 per cent or more was in the form of nicotinuric acid.

Patient McS became ill, complaining of nausea and vomiting, shortly after therapy with nicotinuric acid was begun. As a result of this illness, caloric intake during the first few days of therapy with nicotinuric acid was reduced. It is uncertain whether or not the nicotinuric acid administration was the cause of the gastrointestinal disturbances since the patient was able to continue the medication and, after a few days, again consumed all of the diet. After withdrawal of therapy, the patient was maintained on a low calorie diet which was calculated to be the equivalent of food intake during therapy with nicotinuric acid. During this period, the serum cholesterol returned to control levels. It therefore seems unlikely that the lowered caloric intake was the explanation for the marked decrease in serum cholesterol levels during administration of nicotinuric acid.

COMMENTS

The data presented here, indicating that large doses of nicotinic acid are effective in lowering serum cholesterol levels, are in

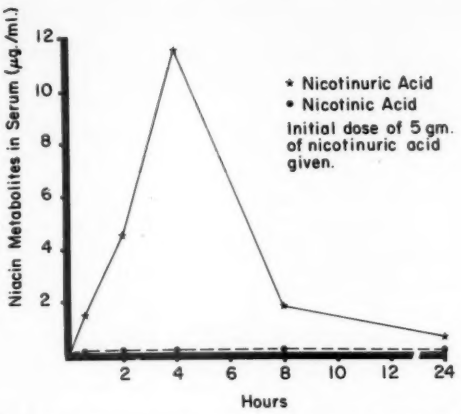

FIG. 11. Concentration of nicotinuric acid and nicotinic acid in serum after oral administration of nicotinuric acid is plotted as a function of time (subject McS).

agreement with those of other investigators.[1–5] Although attempts have been made to study the mechanism by which nicotinic acid lowers serum cholesterol levels, only one possibility seems to have been eliminated by this study. Data presented here and in a previous report[10] demonstrate beyond doubt that nicotinic acid does not lower serum cholesterol levels by increasing the excretion of sterols and bile acids in the feces. Other findings in the current study provide indirect evidence that nicotinic acid does not operate by virtue of some metabolic relationship to one of the lipotropic factors, methionine. Methionine does not negate the effect of nicotinic acid on serum lipids, and nicotinamide, which requires more methyl for excretion than nicotinic acid, does not influence serum lipid levels. Inasmuch as both methionine and choline influence lipid transport, the aforementioned data suggest that nicotinic acid does not influence lipid transport indirectly through methionine.

By a process of elimination it seems likely that the mechanism of action of nicotinic acid is either one of influence on the rate of synthesis of cholesterol or other lipids, or effect on the distribution of lipids between the vascular and the cellular compartments. Evidence for the latter has been suggested by Merrill et al.[15] and by Duncan and Best[16] in their studies with rabbits and rats, respectively. No studies relating nicotinic acid therapy to the synthesis of cholesterol in man have been reported. However, conflicting evidence has appeared in the literature concerning the turnover of cholesterol in experimental animals which had been fed nicotinic acid as compared to animals which had not received this compound. For example, Merrill[17] has reported that nicotinic acid administered to rats causes an increased incorporation of C^{14}-acetate into the cholesterol of the liver. Conversely, Duncan and Best[16] reported that administration of nicotinic acid to rats had no effect on the incorporation of acetate into cholesterol in the liver. Perry,[18] using rat's liver slices, has found that nicotinic acid decreased lipid synthesis. It would seem profitable to study the effect of nicotinic acid on the biosynthesis of cholesterol and other lipids in man.

A recent paper by Friedman and Byers[19] suggests that the effect of nicotinic acid in lowering serum cholesterol levels in man may be due to a decrease in appetite. In our studies with human subjects, controlled dietary regimens have been followed under careful supervision and anorexia has not been noted. Patients have consumed all of the diets prescribed with only minor exceptions. Anorexia may occur in rats but it is not the explanation of the effect of nicotinic acid in man.

In the course of these studies, it became apparent that nicotinuric acid might be the active metabolite of nicotinic acid which is responsible for its action in lowering serum cholesterol levels. The data presented here give strong support to this hypothesis. More extensive studies are planned and are necessary to establish the theory. The studies to date have been hampered by the lack of sufficient amounts of nicotinuric acid, and also by the fact that nicotinuric acid is more slowly absorbed than nicotinic acid and hence much larger doses must be given to obtain the desired effects. Therapy with nicotinuric acid has marked advantages over administration of nicotinic acid because side effects, flushing and pruritis, were not observed during treatment with nicotinuric acid.

It will be necessary to study the effect of nicotinuric acid on each of the possible mech-

anisms involved in lowering serum lipid levels to ascertain the focal point of action.

SUMMARY

Nicotinic acid was found to lower serum lipid concentrations, thereby confirming reports from other laboratories. The mechanism of action of nicotinic acid does not appear to be related to depletion of methyl groups in the body because administration of methionine had no influence on the action of nicotinic acid in lowering serum lipid levels. Nicotinic acid was shown to have no influence on the fecal excretion of bile acids and sterols. Other possible mechanisms of action are discussed.

Evidence is presented which gives strong support to the thesis that nicotinuric acid, a metabolite of nicotinic acid, is responsible for the hypolipemic effect of nicotinic acid.

ACKNOWLEDGMENT

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Experimental Atherosclerosis

Effects of Sulfur Compounds on Hypercholesteremia and Growth in Cysteine-Deficient Monkeys

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A FORM of vascular disease resembling human atherosclerosis has been produced in the New World primate Cebus fatuella.[1] This experimental disease was preceded by a marked elevation of the total cholesterol levels and beta-lipoprotein content of the blood serum. In order to produce these phenomena, the diets had to be rich in cholesterol, choline and neutral fat but relatively low in organic sulfur compounds. Without this deprivation of organic sulfur the response of the serum lipids to cholesterol feeding was small, although definite, and during the periods of observation was not associated with evidences of atherosclerosis. It was shown that supplementation of such diets with either L-cystine or DL-methionine would prevent the marked lipidemia† or, when added after the serum change had appeared, would largely, although not completely, reverse that abnormality. This phenomenon has now been confirmed in mice, rats[2] and chickens.[3,4] However, it was not possible to influence the cholesterol levels during a short term study on ambulatory men treated with 3 gm. of DL-methionine per day.[5] It then became important to investigate the mechanism by which this dietary disturbance of sulfur metabolism influenced sterol metabolism and the integrity of blood vessels.

The similar action of L-cystine and DL-methionine was of immediate pertinence. Cystine has often been considered a non-essential amino acid although capable of sparing the requirement for methionine to some degree.[6] The present experiments were undertaken in an effort to evaluate the relative effectiveness of methionine, cystine and several chemical congeners of these compounds in reversing the hypercholesteremia of monkeys fed diets of fat and cholesterol which were also deficient in sulfur. At the same time two measurements of anabolism, the level of plasma hemoglobin and the body weight were used to interpret the role of these sulfur compounds with respect to the more conventional nutritional requirements of the monkeys. After an interruption of three years these experiments have been repeated with some variations of details of diet. The original results have been confirmed in all details. It has been shown that L-cysteine, L-cystine, DL-methionine, cystamine, glutathione and taurine are each effective in correcting the metabolic disorder produced by such dietary treatment in Cebus monkeys. Cysteic acid has been found inactive.

METHODS

The laboratory management of Cebus mon-

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† Lipidemia is used to mean excessive levels of cholesterol and/or lipoproteins in the blood serum. It is to be distinguished from lipemia which usually means milky or lactescent serum caused by high content of neutral fat.

TABLE I
Composition of Diets and Vitamin Supplements

Item	Diet H	Diet V
Composition of Diet (gm.)		
Sucrose	533	578
α-Protein	200	...
C1-assay protein	...	200
Corn oil	150	...
Lard	...	150
Salts IV	40	40
Cholesterol	50	10
Choline	5	...
Inositol	1	...
p-Aminobenzoic acid	1	...
Vitamin mixture	...	22*
Cod liver oil	20	...
*Vitamin Supplements (mg.)**		
Thiamine	1.0	2.25
Riboflavin	1.0	2.25
Pyridoxine	1.0	2.25
Ca pantothenate	3.0	6.8
Folacin	0.1	0.205
Biotin	0.02	0.045
Niacin	4.9	10.0
Ascorbic	25.0	100.0
Choline	...	170.0
p-Aminobenzoic acid	...	11.5
Inositol	...	11.5
Vitamin B_{12}	...	0.003
Vitamin K	...	5.2
Vitamin E	...	11.5
Vitamin A	...	2,050 units
Vitamin D	...	200 units
Cod liver oil	20	...

* In diet H vitamin supplement was added daily (in 5 ml. 20% ethanol); in diet V it was incorporated in diet (supplying the 50 gm. diet).

keys has been described previously.[7] For the present experiments a group of twenty-four adult and adolescent monkeys of both sexes was used. These animals had been maintained on diet H or diet V (Table I) for four weeks or longer to produce hypercholesteremia of at least 300 mg. per cent. Diet H furnished 300 mg. of L-methionine and 30 mg. of L-cystine per 100 gm. of food. These estimates are based upon the analysis of "α-protein" reported by Grau and Kamei[8] and take into account the average protein content (N × 6.25) as measured by routine analysis of successive batches of the protein. Diet V, prepared with another soya protein, supplied 180 mg. of L-methionine and 108 mg. of L-cystine per 100 gm. of food when calculated with the data from the manufacturer's analyses. The animals were studied in pairs to assess the effects of treatment with the various compounds. These trial groups of animals were composed on the basis of the degree of existing cholesteremia, so that compounds could be compared on similar material. The sulfur compounds were administered orally for periods of twenty-eight days and blood samples were obtained for lipid measurements at seven- or fourteen-day intervals. After completion of such an assay the animals were returned to the basic diet for four weeks or longer until a hypercholesteremia of 300 mg. per cent or more had been restored. They were then used for additional tests. A total of eighty-six such trials have been made with seven different compounds. It is noteworthy that the response of animals to diet V made with lard is essentially the same as that of the earlier studies with diet H made with corn oil. Portman et al.[9] have compared the cholesterolemic effects of lard and corn oil in Cebus monkeys kept on a similar regimen. They concluded that the diets with lard led to higher cholesterol levels, but the variation they found with time makes this conclusion uncertain.

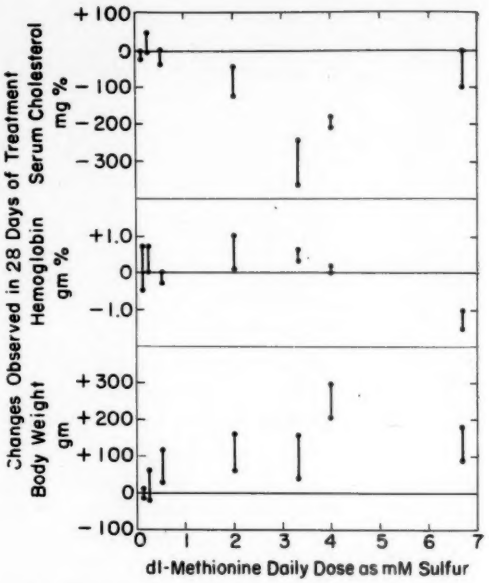

FIG. 1. Effects produced in sulfur-deficient monkeys by supplementation with several levels of DL-methionine. Each point represents the response of one monkey.

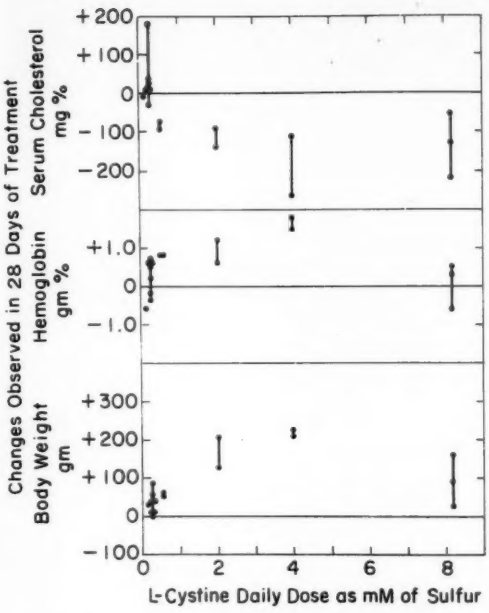

FIG. 2. Effects of supplementation of sulfur-deficient monkeys with L-cystine. Each point represents the effect in twenty-eight days on one monkey.

The serum obtained from the animals was analyzed for total cholesterol by the method of Abell et al.[10] Hematologic measurements were performed by standard clinical methods on freshly drawn blood stabilized with balanced oxalates.

The sulfur compounds studied were, for the most part, commercial products of reagent grade which were characterized by physical properties and, when indicated, by paper chromatography. The compounds were added to the daily food allotment of the animals. Once a day the monkeys were fed as much food as they would consume in two to four hours. All supplements were readily consumed.

Several of the monkeys have been studied at different levels of supplementation to determine the level at which a maximal effect could be obtained. In addition to the changes of serum cholesterol, hemoglobin and body weight which were measured, there were other less objectively measured signs of physiologic effects such as changes in activity, agility, balding and hair growth.

RESULTS

DL-Methionine

This amino acid has been established as a dietary essential for several mammalian species.[6] There is confusion sometimes in the distinction between the essentiality of methionine with respect to functions of the methyl group and the functions of organic sulfur. In the present studies an abundance of choline was available in the diet and a sufficiency of ethanolamine or its precursors is presumed so that the experimental conditions present a deficiency of organic sulfur. The basic diets H and V supplied 300 and 180 mg. of L-methionine and 30 and 10 mg. of L-cystine per 100 gm. diet, respectively. Evidence in chickens and rats[11] indicates a minimal requirement of 0.8 per cent and 0.5 per cent of the diet for these compounds, respectively when methionine comprises a little more than half of the total organic sulfur and when choline is in plentiful supply. It is clear that in actively growing rats and chickens methionine alone will meet the full requirements for organic sulfur. L-Cystine will not meet those requirements under similar conditions although in rats L-cystine may supply as much as 85 per cent of the total requirement of sulfur amino acid.[12]

The responses of fourteen hypercholesteremic monkeys fed DL-methionine at several levels for twenty-eight days is shown in Figure 1. To facilitate comparison of different compounds, the dosage is shown in millimoles of sulfur supplied in the supplement. The growth response passed through a maximum at 4.1 mM of methionine sulfur daily. The reduction in the serum cholesterol level was maximal with a supplement level supplying 3.3 mM of sulfur. At the level of 6.7 mM of sulfur daily the effects both on body weight and cholesterol were less. This effect probably represents an amino acid imbalance, a phenomenon which is well known in growth studies.[13] Small but measureable effects on both weight and cholesterol levels were detected with the administration of as little as 0.55 mM of methionine sulfur daily. Small amounts of sulfur compounds as well as other compounds (to be described) were used in an attempt to find whether there is some low

TABLE II

Comparison of the Effects Produced on the Serum Cholesterol Levels, Body Weight and Hemoglobin Levels by the Treatment of Hypercholesteremic Monkeys with L-Cysteine and Related Compounds for Twenty-Eight Days

Compound (daily mM dose sulfur)	No. of Monkeys	Body Weight (gm.)	Serum Cholesterol Level (mg. %)	Blood Hemoglobin Level (gm. %)
Control	18	−12.22	+20.67	+0.10
L-Cysteine HCl				
2.1	2	−30.0	[−81.5]	−0.60
4.1	2	(+115.0)*	(−229.0)	+0.40
6.3	3	[+48.3]	(−315.0)	+0.60
L-Cysteic acid				
2.1	2	+17.5	−21.5	+0.60
4.0	3	−20.0	+13.3	−0.93
Cystamine†				
2.1	4	[+75.0]	(−127.0)	+0.18
Glutathione (reduced)	2	+27.5	(−169.0)	+0.65

* Comparisons were made between means of the treated groups and the controls by t testing. Probabilities of less than 0.01 for the measurements appear in parentheses and probabilities of less than 0.05 but more than 0.01 for the measurements appear in brackets.

† 2,2′-dithiobis (ethylamine)·2HCl.

level of supplementation which would correct selectively the defect of growth but leave the disturbance of cholesterol metabolism unchanged.

L-Cystine

Eighteen assays were performed with supplementary L-cystine fed at each of eight different levels ranging from 0.12 to 8.2 mM of L-cystine sulfur daily. These data are illustrated in Figure 2. The maximal effect in lowering serum cholesterol levels and in increasing body weight and hemoglobin levels were accomplished by daily supplements with 4.1 mM of cystine sulfur. Furthermore, the magnitudes of the changes induced with equimolar amounts of L-cystine and DL-methionine were similar. It appeared that under these dietary conditions the two compounds were about equally effective in influencing the three variables measured when the compounds were compared on the basis of sulfur content.

L-Cysteine Hydrochloride

Cysteine has been shown to be an effective substitute for cystine in the diets of rats.[14] Seven monkeys were used for study of the effects of three levels of supplementation with cysteine hydrochloride. These data are shown in Table II.

The data for cysteine, cysteic acid, cystamine and glutathione have been evaluated by comparing the changes of weight, total cholesterol levels and hemoglobin levels observed during the period of treatment with the spontaneous changes which occurred for these measurements in eighteen monkeys of similar description, exposed to similar conditions but not treated with sulfur compounds. The data in Table II represent the summaries of these comparisons with the mean changes and the probabilities derived from t tests that these differences exceed the differences observed in the control animals by more than sampling variation.

L-Cysteine hydrochloride produced a significant fall of the serum cholesterol levels and a gain of body weight. The dose of 4.1 mM of sulfur was the most effective in producing growth and both higher and lower doses had lesser effects. The effect on the cholesterol levels was maximal with 6.3 mM of cysteine sulfur per day. The optimal amounts of L-cysteine hydrochloride gave an effect similar in magnitude to that obtained with optimal quantities of either L-cystine or DL-methionine.

Cysteic Acid-Cystamine

Attention was then turned to cysteic acid and cystamine (2,2′-dithiobis [ethylamine]·2HCl),

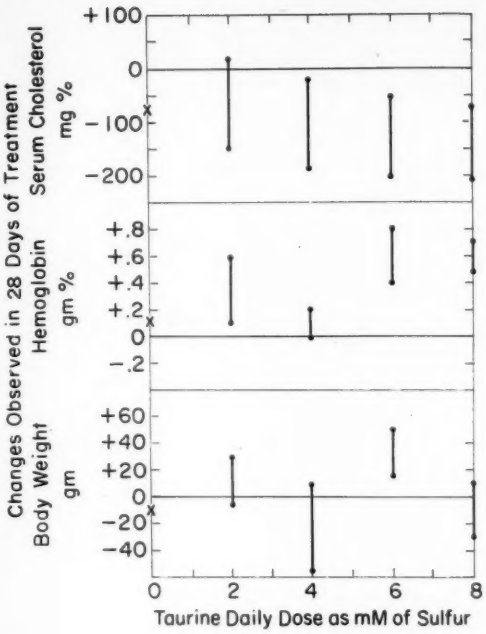

FIG. 3. Effects of taurine supplements on sulfur-deficient monkeys. The initial points (×) represent the mean responses of eighteen untreated monkeys. Each of the other points represents a single treated monkey.

derivatives of cysteine and cystine. Cysteic acid had been studied in sulfur-deficient rats by Andrews[15] and shown[16] to be an ineffective substitute for L-cystine in promoting growth. In trials at two dosage levels with five monkeys, cysteic acid was ineffective in producing change of either body weight or total serum cholesterol level (Table II). The decrease of hemoglobin level produced is of no importance.

The effectiveness of cystamine in promoting growth in sulfur-deficient rats was, for a time, a controversial subject when Sullivan, Hess and Sebrell[17] first reported that the substance would promote growth. In later trials Mitchell[18] and Jackson and Block[19] could find no evidences of growth stimulation in the material. It has been considered that the double decarboxylation of cystine effectively inactivates the compound as a nutrient. Cystamine was studied in four monkeys at a single dose level of 2.1 mM of sulfur per day. This treatment led to a significant increase of body weight and a highly significant decrease of serum cholesterol levels. The hemoglobin level was not changed.

Glutathione

Reduced glutathione was fed to two monkeys at a level equivalent to 2.1 mM of sulfur per day. This treatment (Table II) produced a highly significant reduction of the serum cholesterol, but neither the body weight nor the hemoglobin level was significantly increased. Only one low dosage level was studied.

Taurine

Taurine was of particular interest in this study for several reasons: (1) It comprises a large portion of the urinary sulfur in many mammals.[20] (2) It is involved in sterol metabolism and appears in the bile of several species, including the primates, conjugated by a peptide linkage with the bile acids.[21] (3) It has generally been considered an end product of cysteine metabolism representing a terminal stage of oxidation which makes it irretrievable for the sulfur economy.[22] The presence of free taurine in the tissues of certain species[23] and the incorporation of radiotaurine into mammalian tissues[24] suggested, however, that taurine may not be as physiologically inert as had been supposed. The present data supply additional evidence for the physiologic importance of taurine.

Twelve monkeys have been studied at four levels of administration of taurine. The relation of the response to the dose administered was different from that observed with the physiologically active compounds previously tested (Fig. 3). Comparison of the cholesterol levels at each interval when blood samples were tested with the control level and the pooling of data from all doses indicated that treatment with taurine significantly lowered these levels at each interval. A maximal effect was produced by the fourteenth day of treatment and the effect diminished thereafter. Thus, taurine resembled L-cystine, DL-methionine and L-cysteine hydrochloride in producing a significant reduction in hypercholesteremia. Administration of taurine did not stimulate growth in these studies. Since the net effect of taurine was less than that of the other active

compounds and did not produce evidences of amino acid imbalance at high doses, the possibility of the presence of one of these active compounds as a contaminant in the taurine was considered. The recrystallized taurine used was analyzed by the Brand modification of the Sullivan reaction.[25] This procedure revealed the presence of 0.24 per cent of material reacting as cystine. This amount of contamination would comprise less than 0.01 mM of cystine sulfur in the taurine when this was fed at a level of 4 mM of sulfur. Previous experience with cystine (Fig. 2) indicated that would be an ineffective dose. Paper chromatography* [26] also failed to reveal significant contamination of the taurine by other sulfur compounds.

COMMENTS

After a few trials it was clear that there was some relation between the degree of elevation of the serum cholesterol and the ease with which it would be influenced. This phenomenon appears to be similar to the relation between the spontaneous variability and the level of serum cholesterol and beta-lipoprotein in human beings described by Watkin et al.[27] Careful matching of the assay groups of animals has minimized the importance of this influence in the present work. A computation of these assay data according to millimoles of sulfur administered per unit of initial body weight of the animals did not significantly alter the conclusions that were drawn. The average body weight of the animals was 1,250 gm.

The equivalence of equimolar amounts of sulfur supplied as DL-methionine, L-cystine and L-cysteine hydrochloride in lowering hypercholesteremia and promoting growth in these monkeys indicates that the effect was produced by cysteine or by some metabolite of cysteine. Each of these compounds would be expected to supply cysteine as would glutathione, which was also active. The activity of a racemic mixture of methionine was expected in view of the observations of several workers with a variety of species which indicate that the D-isomer of methionine is efficiently used.[28–30] This utilization of a D-isomer may be accounted for as shown in the demonstration by Binkley[31] that in the conversion of methionine to cysteine only the sulfur is supplied by methionine, the carbon moiety arising from the contribution of serine to the intermediate compounds, cystathionine.

The effects of cystamine and cysteic acid observed herein reveal insight into the mechanism of the sterol defect in the monkeys. Cysteic acid will neither promote growth in sulfur-deficient rats or monkeys nor will it lower the cholesterol level of these cysteine-deficient monkeys. However, Virtue and Doster-Virtue[32] observed that cysteic acid was a potent stimulator for the production of taurocholic acid in bile-cannulated dogs. This was interpreted to indicate that cysteic acid is readily converted to taurine in the dog and a cysteic acid decarboxylase, which appears to facilitate this reaction, has been demonstrated.[33] Cystamine was an effective compound in reversing the hypercholesteremia of monkeys as well as promoting growth. This growth stimulation is in agreement with the early work of Sullivan, Hess and Sebrell[17] which was later disproved by the work of other laboratories in which cystamine was found to be inactive. Virtue and Doster-Virtue have found cystamine ineffective in stimulating the production of taurocholate in dogs.[34] The metabolic significance of cystamine is thus thoroughly confused. The compound cannot be considered a precursor of cysteine. It is plausible that cystamine might act as a precursor of thiolethanolamine. Although thiolethanolamine is a component of coenzyme A, Hoagland and Novelli[35] found it to be an ineffective source of sulfur with an *in vitro* system for pantethiene synthesis. This observation, *inter alia*, led those authors to conclude that pantotheine is formed from cysteine and pantothenate. The activity of thiolethanolamine was not studied in these experiments.

The activity of taurine observed in the present studies suggests two possible explanations. Taurine may be an obligatory conjugate in the metabolism of sterols, particularly in the conversion of cholesterol to bile acids.[36] In the presence of a shortage of organic sulfur compounds exogenous taurine would then restore

* Carried out by Dr. Oscar Portman with the technic of Bentley and Whitehead.[26]

sterol metabolism but would not restore growth and the other actions of organic sulfur compounds which are less completely oxidized. Alternatively, taurine may act indirectly by diminishing a channel of waste of sulfur. For example, if cysteine wastes to taurine, an exogenous supply of taurine may diminish this process and thus conserve cysteine for the reaction affecting sterol metabolism. The unique dosage-response relation of taurine does not seem to help in resolving this alternative.

Attempts were made to find compounds and dosage levels which would discriminate between the growth (as measured by body weight and hemoglobin regeneration) and the effect on sterol metabolism. If dissociation could be demonstrated, it would materially simplify the problem of the mechanism of the interrelation of sulfur and cholesterol metabolism. The dissociation could only be demonstrated with taurine. The responses to administration of this compound suggest that normal cholesterol metabolism requires cysteine or certain of its metabolic intermediaries which are not essential or effective in more general anabolic processes.

SUMMARY

The effectiveness of L-cystine, DL-methionine, L-cysteine hydrochloride, L-cysteic acid, cystamine, taurine and reduced glutathione in stimulating growth, production of hemoglobin and reduction in hypercholesteremia of sulfur-deficient monkeys has been studied. These compounds, with the exception of cysteic acid, are shown to be effective. The activities of the compounds are approximately equivalent when compared on a mole sulfur basis except for taurine, which is less effective than the other compounds on a mole sulfur basis, and while it reduces the hypercholesteremia it does not stimulate growth. The relation of these findings to sterol and sulfur metabolism is discussed.

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Studies of Adipose Tissue in Man

A Microtechnic for Sampling and Analysis

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ABUNDANT evidence may be found indicating that adipose tissue plays a central role in many phases of lipid and carbohydrate metabolism.[1] Yet, due to analytic limitations as well as to the necessity for surgical excision of tissue, there have been few biochemical studies of this tissue in man. With the advent of gas-liquid chromatography (GLC), precise analyses of fatty acid composition can now be completed with less than a milligram of fat. The present report describes a simple, virtually painless and risk-free method for the removal of such small samples of adipose tissue.[2] Results obtained by the use of this method of adipose sampling will be presented and discussed.

METHODS

Obtaining the Sample

Samples of adipose tissue are aspirated with ease from any subcutaneous area over the trunk or extremities, but the buttock is usually the most convenient site. After preliminary procainization of the skin, a No. 18 thin-walled needle with stylus (T 462 LNR, Becton, Dickinson and Co., Rutherford, New Jersey) is inserted through the procaine wheal 2 to 3 cm. into the subcutaneous adipose layer. The stylus is removed and about 1 ml. of isotonic saline is injected through the needle from a 50 ml. standard venepuncture syringe. Maximum traction on the syringe plunger is applied, and while suction is maintained, the syringe and needle are repeatedly rotated and pushed back and forth in the adipose tissue. These movements are most easily accomplished by grasping the skin and adipose layer between the thumb and forefinger of one hand and the syringe barrel, with retracted plunger, in the other. In this manner a portion of the injected saline is recovered along with numerous minute shiny fat droplets which cling to the inner surface of the syringe. If the first attempt is unproductive, the aspiration can be repeated without relocating the needle or injecting more saline. This simple technic is illustrated in Figure 1.

FIG. 1. The technic of adipose aspiration is illustrated by this diagram showing retraction of the syringe plunger and withdrawal of fat droplets from the subcutaneous adipose tissue.

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Preparation and Analysis of the Sample

The entire aspirate is quantitatively transferred from the syringe barrel into a 60 ml. glass-stoppered bottle by washing the syringe interior and plunger with 20 ml. of a 1:1 (v:v) solution of isopropyl alcohol and petroleum ether (30 to 60° c.).

Removal of non-lipid substances such as protein and salt is accomplished as follows: The extract is filtered through ether-treated shark-skin filter paper into a 60 ml. separatory funnel moistened with distilled water. Then the filtrate is washed twice by shaking with 15 ml. of distilled water. At each step the lower phase is discarded, and the final washed upper phase is decanted into a 60 ml. glass-stoppered bottle containing 2 to 4 gm. of anhydrous sodium sulfate. The final volume of the extract is close to 10 ml., and from this a 1 or 2 ml. aliquot is removed for gravimetric determination of total lipid.[3] The weighed solute is discarded. An appropriate portion of the remaining solution (equivalent to 0.2 to 5 mg. of triglyceride) is removed for formation of methyl esters by interesterification with anhydrous methanol.[4] The mixture of methyl esters is then analyzed by a standard technic of GLC. The linearity of detector response of our chromatographic device has been rigorously tested;[5] this is essential to the success of GLC as a quantitative technic.

For identification and accurate measurement of fatty acid composition, elutions are carried out both on polar (ethylene glycol adipate polyester) and non-polar (Apiezon-M) stationary phases at 184.5° and 197° c., respectively. The combined use of polar and non-polar stationary phases enables the most precise resolution of components from eight to twenty-two carbon atoms in length, but for simpler comparative studies of adipose composition, runs are often made on polar columns alone. However, in the latter case the oleic acid isomers are not resolved from the larger oleic acid (18:1) peak. Furthermore, measurements of 16:1, 14:0 and 12:0 are more accurate when obtained by a two-column analysis.

Precautions

All solvents are reagent grade and redistilled in an all glass apparatus. Needles, syringes and glassware are chemically cleaned and extracted in organic solvents to remove all traces of fatty contaminants. Rubber stoppers, plastics, Luer-lock or other devices glued to the syringe should not be used at any stage, since organic solvents may extract interfering substances from such materials. Hence, disposable syringes and needles are unsatisfactory. The aspirate may be stored in the initial extraction solution for many weeks. However, the risk of fatty acid oxidation and other chemical changes increases with time. Therefore, it is wise to complete the further purification and analysis as quickly as possible. In order to avoid oxidative changes, DL-α-tocopherol has recently been added to the isopropyl alcohol-petroleum ether extracting solution (1 μL. per 20 ml.). Whether or not this will prevent all oxidative artifacts is not yet known.

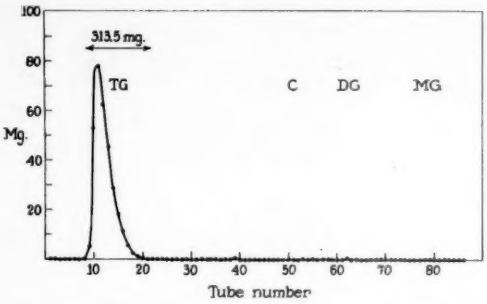

FIG. 2. Silicic acid chromatography of 313.6 mg. of adipose lipid (gradient elution with ethyl ether in petroleum ether). The elution of triglycerides (TG) from an 18-gram column of silicic acid by gradient elution.[6] The absence of components other than triglycerides is evident. (C = cholesterol, DG = diglyceride, MG = monoglyceride.)

In deteriorated samples, new unidentified peaks appear on the chromatograms as unsaturated components progressively diminish. On adipate columns at 184.5° c., these new peaks have adjusted retention times of 7.40 and 7.00 relative to methyl stearate, and less frequently there are peaks at 0.945, 2.82 and 3.92. On Apiezon columns, an artifactual component precedes 12:0 with a retention time of 0.050 relative to methyl stearate. It is hoped that the addition of tocopherol to the extraction medium and the prompt processing of all sam-

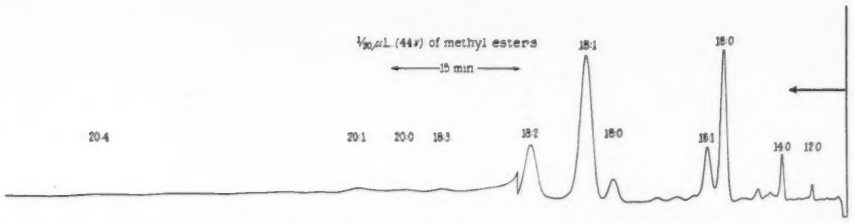

FIG. 3. Gas-liquid chromatogram (EGA polyester) of the methyl esters of normal adipose tissue at 180° c. with origin at right margin. Major peaks are labeled by chain length (number before colon) and by double bonds (number after colon).

ples will prevent the occurrence of these artifacts. When more than a trace of these components is noted, the samples must be discarded.

Subjects

In the studies to be described 145 subjects have been examined. These include healthy medical students and laboratory staff, as well as hospital in- and outpatients with a variety of clinical disorders. In many instances hospital patients on special diets were studied. These diets were administered as liquid formulas prepared from egg white, dextrose and a single fat. This technic has been described in detail elsewhere.[6] A few special analyses of fat removed at autopsy or during surgery were obtained through the courtesy of the Departments of Pathology and Surgery of the New York Hospital-Cornell Medical Center. The Departments of Pathology, Pediatrics and Obstetrics at the New York University-Bellevue Medical Center and Columbia-Presbyterian Medical Center have also made certain subjects available for study.

In many subjects blood was drawn at the time of biopsy of adipose tissue. Serum lipids were extracted by the method of Folch,[7] and the three major lipid ester classes were then separated chromatographically on columns of silicic acid.[8] Finally, fatty acid analysis of each ester class was performed by GLC. The small fraction of free or non-esterified fatty acids was separated by titration from another aliquot of serum by the method of Dole.[9] A sufficient quantity of these acids could be titrated from 10 to 15 ml. of serum to permit satisfactory GLC analysis. Since this extract is contaminated with traces of phospholipid (phosphatidyl serine and possibly other polar lipids are titratable under these conditions), the fatty acids were freed from these contaminants by passage over several grams of silicic acid in ethyl ether. Such batch treatment is highly effective in the differential removal of phospholipids from complex lipid mixtures.

RESULTS

To date, 391 adipose aspirations have been performed on 145 subjects. The procedure has been found completely free of complications. It is to be expected, however, that cases of procaine sensitivity or adverse psychic reaction may be encountered. A brief preliminary questioning reduces the likelihood of such occurrences.

Lipid Constituents of Adipose Tissue

The yield of lipid has averaged 3.68 mg. per aspiration with a range of zero to 34.3 mg. Since aspirations may be repeated with ease, it is feasible to remove as much as 25 mg. from a single subject within ten to twenty-five minutes. The lipid removed from adult adipose tissue is more than 99 per cent triglyceride. Determinations carried out on large pooled samples of aspirate revealed only 0.3 per cent total cholesterol and less than 0.1 per cent phospholipid. When a single, large sample of adipose tissue from the anterior abdominal wall was obtained surgically and promptly extracted and analyzed by silicic acid chromatography, it was found that the large non-phospholipid, non-sterol moiety was exclusively triglyceride. Figure 2 shows that 313.5 mg. of the 313.6 mg. applied was found in the triglyceride peak. If unesterified cholesterol (C), diglyceride (DG) or monoglyceride (MG)

TABLE I

Micro- vs. Macro-Sampling of Subcutaneous Adipose Tissue

(Comparison of Data Obtained at the Same Anatomic Site (Abdominal Panniculus) by Needle Aspiration and by Surgical Excision of Superficial and Deep Adipose Layers)

Fatty acid	Needle aspiration	Surgical excision	
		Superficial	Deep
14:0	2.6	2.1	2.3
16:0	23.3	22.4	23.0
16:1	5.4	4.3	4.3
18:0	4.6	5.7	4.8
18:1*	47.7	50.4	51.7
18:2	9.3	10.7	9.0

* This includes the isomers of 18:1.

had been present, they would have appeared in the designated locations. In contrast to adult adipose tissue, subcutaneous fat removed at the autopsy of a premature, stillborn infant contained 2.2 per cent phospholipid and 1.2 per cent total cholesterol. By histologic examination this specimen was seen to be far more cellular, with less of the signet-ring cell appearance characteristic of adult adipose tissue.

When adipose triglyceride is interesterified with methanol, the resultant fatty acid methyl esters are separated and can be identified as shown in the gas-liquid chromatogram of Figure 3. The major peaks are labeled as to chain length and content of double bonds. The predominance of 16:0 (palmitic) and 18:1 (oleic) acids is evident, but a large number of lesser components can also be seen. In all, there may be as many as thirty-five or forty peaks, but six major acids always comprise more than 90 per cent of the total.

Validation of Aspiration Technic

A comparison of the concentrations of the six major fatty acids in tissue removed by various technics and from different sites has served to validate this method of adipose sampling. In Table I the concentrations of these six acids (myristic, palmitic, palmitoleic, stearic, oleic and linoleic) found by percutaneous aspiration from the anterior abdominal wall are compared with analyses made on tissues removed surgically from the superficial and deep portions of the subcutaneous fat layer at the same site as the needle aspiration. The similarity of results indicates that the aspiration method of adipose removal provides a representative sample of tissue. Table II compares the fatty acid compositions of aspirates from different superficial sites in the same subject. With the possible exception of the 16:1 and 18:0 acids, the differences seem negligible. In Table III a sample aspirated at autopsy from the subcutaneous fat of the buttock is compared with fatty tissue removed from between the fasciculi of the psoas muscle and also from the omentum, perinephric area and pericardium. Although the fatty acid composition of omental fat is practically indistinguishable from that of the needle aspirate, the other deep sites show slight changes which are most pronounced in the pericardial sample. In this location there is a definite, although small, decrease in C_{18} unsaturated acids.

TABLE II

Similarity of Fatty Acid Structure at Various Subcutaneous Sites

(Comparison of the Fatty Acid Structure of Adipose Tissue Removed by Needle Aspiration from Different Subcutaneous Sites in the Same Subject)

Fatty Acid	Site				
	Buttock	Abdomen	Thigh	Arm	Interscapular Area
14:0	2.0	2.6	2.0	2.1	1.9
16:0	22.7	23.3	21.1	22.3	22.6
16:1	6.2	5.4	5.8	4.4	3.9
18:0	3.7	4.6	4.3	5.7	6.7
18:1*	49.3	47.7	50.0	49.0	49.3
18:2	9.9	9.3	10.3	10.3	10.7

* This includes the isomers of 18:1.

It is clear that a needle aspirate of subcutaneous fat provides a highly representative sample of the adipose fatty acids in all regions of the body, as well as a representative sample of the fatty acids present locally. In many animal species, superficial adipose fat is more unsaturated than the fat of deeper, internal depots.[10] However, as Cuthbertson and Tompsett noted earlier,[11] these differences are negligible in man. This uniformity in composition of human depot fat is presumably the result of

TABLE III

Superficial vs. Deep Adipose Tissues
(Comparison of Adipose Fatty Acid Composition in Fatty Tissue Removed from a Single Superficial and Four Deep Sites in the Same Subject)

Fatty Acid	Needle Aspiration	Excision			
	Buttock	Psoas Muscle	Omentum	Perinephric Area	Pericardium
14:0	2.0	2.8	2.6	1.9	3.1
16:0	22.7	25.4	20.5	20.1	27.2
16:1	6.2	4.8	7.0	4.3	6.8
18:0	3.7	4.7	4.8	6.5	4.4
18:1*	49.3	48.5	49.0	51.4	45.4
18:2	9.9	8.9	10.8	10.3	8.5

* This includes the isomers of 18:1.

wearing clothes and taking other measures to maintain a warm skin, since animals grown in warm environments show similar uniformity in composition.[12]

Normal Fatty Acid Composition of Subcutaneous Adipose Tissue

Having examined the validity of this technic, it was thought essential to establish a set of values which might be considered as the "normal" fatty acid composition. The selection of a normal composition is highly arbitrary for, as will be shown, dietary alterations leave an imprint on adipose composition without in any way rendering it abnormal. Furthermore, newly born premature and full term infants have different compositions of adipose tissue, neither of which can be said to be abnormal. To designate these patterns as normal serves only to provide a standard or reference for relating adipose and other compartments of lipids and for the study of changes in adipose tissue as a function of age, metabolic state, disease and diet.

To establish a reference composition, twelve healthy subjects between the ages of twenty and thirty-five were selected. All were on random diets and gave no history suggesting dietary peculiarities, metabolic disease or recent change of weight. The means and standard deviations of fatty acid concentrations in these subjects are shown in Table IV. The twenty-two acids listed comprise 98 per cent of the total; however, it is notable that seven acids account for 92 per cent. An additional group of acids of uncertain identity which occur occasionally and in only trace amounts have

TABLE IV

Fatty Acid Analyses of Subcutaneous Adipose Tissues Obtained by Needle Aspiration from Normal Subjects Between the Ages of Twenty and Thirty-Five Years (Five Women and Seven Men)

Fatty Acid	Mean ± Standard Deviation
12:0	0.7 ± 0.1
14:0	3.3 ± 0.1
14:1	0.6 ± 0.05
14:0 branched*	0.1 ± 0.1
15:0	0.6 ± 0.1
15:0 branched*	0.3 ± 0.3
16:0	19.5 ± 2.1
16:1	6.9 ± 0.1
17:0	0.2 ± 0.2
17:0 branched*	1.0 ± 0.3
18:0	4.2 ± 1.1
18:1	41.2 ± 4.4
18:1 isomers*	5.1 ± 1.0
18:2	11.4 ± 1.4
18:3	0.4 ± 0.1
19:0 branched*	0.5 ± 0.2
20:0	0.6 ± 0.1
20:1	0.6 ± 0.3
20:2	0.1 ± 0.1
20:2*	0.1 ± 0.1
20:3	0.2 ± 0.1
20:4	0.2 ± 0.1

NOTE: Underlined components comprise 92 per cent of the total mixture.

* Identity not established.

TABLE V
Fatty Acid Composition of Human Adipose Tissue (Results by Present Technic and by Classic Methods)

Fatty Acid	Needle Aspiration and GLC Analysis (12 samples), 1960	Autopsy Specimens—Classic Methods (5 samples), 1943
12:0	0.7	0.5
14:0	3.3	3.5
14:1	0.6	0.4
16:0	19.5	25.0
16:1	6.9	6.4
18:0	4.2	7.0
18:1*	46.3	45.9
18:2	11.4	9.6
20:4	0.2	0.7
Other 20 carbon acids	1.7	1.2
Total	94.8	100.2

NOTE: The means of twelve "normal" samples obtained by the present technic are compared with the means of five autopsy samples analyzed by classic methods.[13] Note that acids with an odd number of carbon atoms and other small components are not listed among the results obtained by the new method. This accounts for the difference in the two totals.

* This includes the isomers of 18:1.

not been listed; these account for the remaining 2 per cent. Only negligible amounts of acids with chain length greater than twenty carbon atoms are seen. The high standard deviations of some of these acids most likely express interindividual differences and are not attributable to the analytic methods employed, for it has been shown[5] that replicate analyses of a given mixture by GLC are accomplished with standard deviations less than 1.5 per cent of the mean.

The "normal" adipose composition shown in Table IV corresponds well with the analyses made over fifteen years ago by Cramer and Brown.[13] Using fractional distillation and low temperature crystallization, they analyzed five samples of human adipose tissue; Table V presents the mean of these data. It is not clear whether the small differences in these two sets of data are of analytical origin or truly represent differences in the adipose tissues analyzed.

Comparison with the Fatty Acids of Serum

It is not possible to provide an exact statement of the metabolic relation of adipose tissue to any serum lipid compartment by simple comparisons of fatty acid compositions of these entities. Nevertheless, such comparisons may suggest certain important interrelations. In Figures 4 and 5 the adipose composition (as indicated by a bar graph of seven acids: 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 20:4) of a healthy twenty-two year old man on a random diet is compared with the composition of four serum lipid classes. Both adipose and serum samples were obtained at 8:00 A.M. following a twelve-hour fast. Although the adipose composition is seen to be practically indistinguishable from the reference values of Table IV, it is noteworthy that no serum lipid group is precisely equivalent to adipose tissue in its array of fatty acids. The triglyceride and non-esterified fatty acids show the closest resemblance

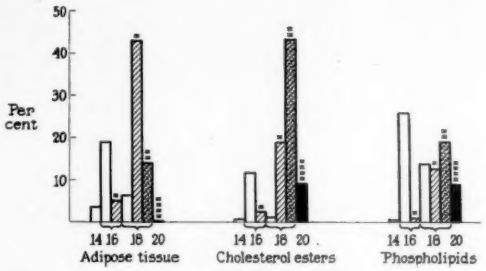

FIG. 4. Fatty acid composition of adipose tissue and serum lipids (random diet) compared with serum cholesterol ester and phospholipid fatty acids. Chain length and number of double bonds of each acid are indicated. The individual studied had been on a random diet with no history of metabolic or dietary abnormalities.

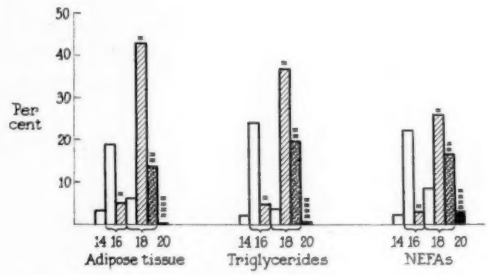

FIG. 5. Fatty acid composition of adipose tissue and serum lipids (random diet) with serum triglyceride and non-esterified fatty acids (NEFA's) in the same subject as in Figure 4.

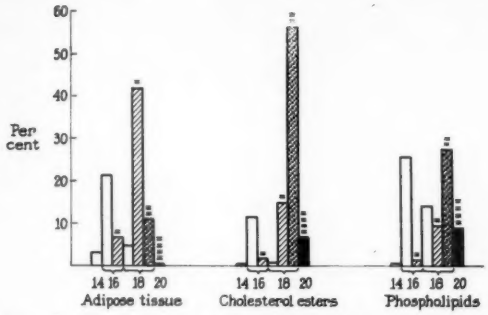

FIG. 6. Fatty acid composition of adipose tissue and serum lipids (corn oil diet) compared with serum cholesterol ester and phospholipid fatty acids. This subject had been on a diet containing 40 per cent of calories as corn oil (linoleic acid = 54 per cent) for ten weeks. Note the large amount of linoleic acid ($18^{=}_{-}$) in the serum fractions.

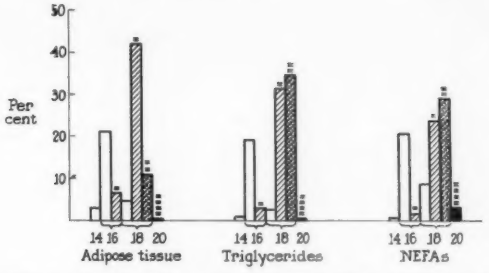

FIG. 7. Fatty acid composition of adipose tissue and serum lipids (corn oil diet). The fatty acids of serum triglycerides and non-esterified fatty acids (NEFA's) compared with adipose tissue in the same subject shown in Figure 6.

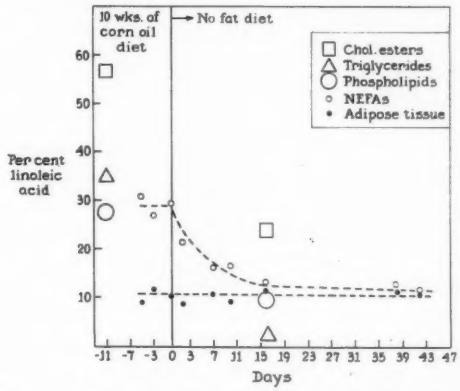

FIG. 8. Dietary effects on the linoleic acid (18:2) content of adipose tissue and four serum lipid fractions. A diet rich in corn oil (40 per cent of calories) was changed isocalorically to a fat-free diet without altering protein or total calories. Note the rapid changes in all serum compartments, but not in adipose tissue.

to adipose tissue, but even these contain far larger amounts of 18:2 and 20:4.

It would appear that no serum lipid group derives its fatty acids from the adipose tissue in a simple, unselected way. More likely, the serum lipids represent mixtures of acids of two origins, dietary and adipose fat. As dietary and metabolic situations vary, either the diet or adipose tissue may become the primary source of serum fatty acids. Although these changing circumstances are most quickly reflected in the triglyceride and non-esterified fatty acids, the composition of cholesterol esters and phospholipids is also involved. Thus, Figures 6 and 7 compare the adipose and serum lipid constituents after ten weeks of corn oil feeding in a thirty-four year old man with hypercholesterolemia, in whom one-fifth of total calories was derived from linoleic acid. Even though each of the serum lipids reflected this high intake of 18:2, the composition of the adipose tissue remained unchanged. When the regimen was changed to a fat-free and hence linoleic acid-free diet by substituting carbohydrate for fat calories, the linoleic acid content of the serum non-esterified fatty acids quickly fell to the level present in adipose tissue (Fig. 8). All other serum lipid compartments also diminished in linoleic acid content. It would appear that adipose tissue becomes a major source of circulating fatty acids when the diet is fat free. Although other possible sources of plasma lipid (such as hepatic lipogenesis) must be considered as well, it appears that adipose fat stands in readiness to provide a sustained fundamental pattern for serum lipid components, whereas dietary fat contributes inconstantly. Thus, knowledge of adipose fatty acid composition would seem to be of paramount significance in the study of fatty acid metabolism in man. Subsequent sections will examine certain changes in adipose composition as a function of age, metabolic state, disease and diet.

Changes With Age

Most of the samples have been from subjects ranging in age from twenty to sixty years.

TABLE VI
Change in Adipose Composition with Age*

Fatty Acid	Subject				
	Four Premature Infants (1,500, 1,610, 1,733, 1,760 gm.)	Three Full Term Infants (4,545, 3,778, 4,100 gm.)	Two Children (8 and 11 yr.)—Mild Rheumatic Fever	Twelve Normal Adults (20 to 35 yr.)	Three Normal Adults (46, 56 and 60 yr.)
14:0	3.7 ± 0.4	3.0	3.7 ± 0	3.3 ± 0.1	2.3 ± 0.2
16:0	26.7 ± 4.4	40.2 ± 0.9	17.1	19.5 ± 2.1	21.2
16:1	10.0 ± 1.4	14.6 ± 0.3	9.8 ± 1.5	6.9 ± 0.1	6.7
18:0	8.4 ± 2.3	5.1	4.7	4.2 ± 1.1	4.0
18:1	26.4 ± 2.7	25.2 ± 1.3	41.4	41.2 ± 4.4	46.2
18:2	12.0 ± 1.0	1.3 ± 0.1	12.8	11.4 ± 1.4	11.5
18:3	0.2 ± 0.2	1.8 ± 0.2	0.6	0.4 ± 0.1	0
20:3	1.2 ± 0.8	3.9 ± 2.5	0.2	0.2 ± 0.1	0.3
20:4	0.9 ± 0.6	0.3	0.2	0.2 ± 0.1	0.3

* All figures are means. Standard deviations are included when means show interesting variations.

In this group we have seen no variations in adipose fatty acid which can be correlated with age. However, newly born premature and full term infants show startling differences from the normal adult pattern. These are illustrated in Table VI. The first column (adipose composition of four premature infants) shows higher concentrations of 16:0, 16:1, 18:0, 20:3 and 20:4 than seen in normal young adults (column 4), but there is a much lower concentration of 18:1. Even more striking changes are seen in three full term newly born infants (column 2) in whose adipose tissue linoleic acid (18:2) has nearly disappeared, while contents of 16:0, 16:1 and 20:3 are even larger than in the premature infants.

It might be expected that progressive development of the fetus in the last trimester of pregnancy would be associated with adipose changes more closely approximating the average adult pattern. Yet, the full term infant shows greater adipose differences from normal than does the premature. It is interesting to speculate on possible explanations for this finding. The accumulation of fat in fetal adipose depots is known to occur primarily toward the end of pregnancy.[14] The premature and full term infants described in Table VI weighed an average of 1,651 gm. and 4,141 gm., respectively; but it is estimated[14] that this threefold change in total body weight parallels an almost twelvefold change in fat content from roughly 75 to 875 gm. Although there is little doubt that some fatty acids can pass from the maternal circulation to the fetus,[15] there is also good evidence that most fetal fat is synthesized *in situ*.[16] The present findings support this view. The similarity of data from the premature infants and young adults, especially the 18:2 contents of the two groups, suggests that early in pregnancy fetal adipose fatty acids originate from a maternal source. But late in pregnancy, lipogenesis from carbohydrate may predominate, in which case a decrease in 18:2 and increase in 16:0 and 16:1 would be expected. These same findings (increasing 16:0 and 16:1 and decreasing 18:2) occur in the rat fed a high carbohydrate, nearly fat-free diet.[17] Evidently, calories in excess of those required for fetal growth are available for lipogenesis late in gestation. Perhaps the enzymatic apparatus responsible for the conversion of carbohydrate to fat in adipose tissue matures late in pregnancy, giving rise to this sudden burst of fat production. In any event, the fat present at term shows a pattern characteristic of those fatty acids which are synthesized from carbohydrate.

The presence of larger than normal amounts of 20:3 in full term infants should not be overlooked. In rats with essential fatty acid deficiency 5,8,11-eicosatrienoic acid is formed in

TABLE VII
Adipose Composition in Various Clinical States

Fatty Acid	State and Subject					
	Normal: 5 Women; 7 Men (20–35 yr.)	Acute Myocardial Infarction: 5 Men (32–65 yr.)	Postpartum State: 3 Women (20–35 yr.)	Obesity: Woman, 58 yr.; 114 kg.	Diabetes: 32 year old Woman; Insulin 40 units/day for 1½ yr.	Wilson's Disease: 39 year old Woman
14:0	3.3 ± 0.1	3.3	1.8 ± 0*	2.4	2.9	2.0
16:0	19.5 ± 2.1	25.0 ± 0.9*	20.6	25.0	18.9	18.0
16:1	6.9 ± 0.1	4.9 ± 0.8*	6.7	6.9	9.8	12.7
18:0	4.2 ± 1.1	4.9	5.5	3.4	4.7	1.9
18:1	41.2 ± 4.4	44.8	45.4	43.6	47.9	42.4
18:2	11.4 ± 1.4	9.0 ± 0.4*	12.1	9.1	11.0	11.8
18:3	0.4 ± 0.1	0.2	0.3	0.3	0.2	0.2
20:3	0.2 ± 0.1	0.1	0.2	0.3	0.1	0.4
20:4	0.2 ± 0.1	0.3	0.5 ± 0.5*	0.5	0.0	0.4

* Standard deviations included when means appear appreciably different from normal values.

excess from oleic acid;[18] indeed a rise in concentration of this C_{20}-triene is one of the earliest biochemical manifestations of this deficiency state.[19] It remains for future studies to determine whether or not the chemical structure of this 20:3 acid in full term infant adipose tissue is the same as that in the weanling rat fed a fat-free diet.

Effects of Various Clinical Situations

There is reason to believe that some disorder of adipose tissue may play an important role in several clinical states. Thus, in obesity the size of the adipose tissue is the chief abnormality, even if the etiologic mechanisms are found elsewhere. Since adipose tissue is a primary locus of insulin action,[1] diabetes also may be looked on as a disorder with an important biochemical expression in adipose tissue. Yet, in several cases of obesity and of diabetes, no significant alterations in the fatty acid composition of adipose tissue were found. An example of each is given in Table VII.

Table VII lists data obtained from five men dying within several days after suffering acute myocardial infarctions. The small changes seen in these acutely ill patients, as well as in the chronically bed-ridden patient with Wilson's disease, are not as yet meaningful. It may be that, when more information on adipose composition has been gathered, such small changes will be interpretable and helpful in the further understanding of these disorders.

The unexpected finding of low linoleic acid in full term infants poses the question of what differences in adipose tissue composition there may be in parturient women. However, Table VII shows no significant abnormalities of 18:2, 16:0 or 16:1 content in postpartum women.

A small group of patients with specific disorders of lipid metabolism (hyperlipemia, hypercholesterolemic xanthomatosis, and Von Gierke's disease with hyperlipemia) have been studied, but no systematic changes typical of these disease entities have been found. In these situations, the antecedent dietary therapy has usually left specific marks on adipose composition (Table VIII). The hypercholesterolemic subject consuming linseed oil as a major source of fat calories showed changes in adipose fatty acids which would be expected with a slow exchange between a typical mixture of adipose fatty acids and that found in linseed oil itself. A similar exchange occurred in the hyperlipemic patient fed a diet rich in corn oil (18:2 = 54 per cent). In the child with glycogenosis who had required high carbohydrate feedings for over five years, a mixture low in 18:2 and high in 16:0 and 16:1 was encountered,

TABLE VIII
Change in Adipose Composition with Diet

Fatty Acid	Diet and Subject					
	Random Diet: 12 Normal Adults, 20–35 yr. (mean ± S.D.)	Linseed Oil, 3 oz./day for 1 yr.: Hypercholesteremic Man, 44 yr. with Arteriosclerotic Heart Disease		Corn Oil, 2 oz./day for 2 yr.: Hyperlipemic Man, 57 yr.		High Carbohydrate, Low Fat Diet for 5 yr.: Glycogenosis (Von Gierke's Disease): 6 yr. old Male Child
14:0	3.3 ± 0.1	1.2		0.7	(0.3)†	3.1
16:0	19.5 ± 2.1	14.7	(5.4)*	15.9	(12.7)†	22.6
16:1	6.9 ± 0.1	5.8		5.8		22.1
18:0	4.2 ± 1.1	5.4	(4.0)*	2.2	(2.7)†	1.0
18:1	41.2 ± 4.4	35.5	(22.6)*	44.0	(30.7)†	43.1
18:2	11.4 ± 1.4	20.5	(16.4)*	25.9	(53.5)†	3.3
18:3	0.4 ± 0.1	13.7	(51.1)*	0.8		0.3

* Fatty acid composition of linseed oil.
† Fatty acid composition of corn oil.

as in the case of full term infants. However, in this instance, the 20:3 acid was not significantly increased.

These three cases emphasize the importance of long standing dietary effects in shaping the composition of adipose tissue. The subsequent section considers these effects in further detail.

Effects of Diet

In adults in caloric balance, dietary effects on adipose tissue are produced only slowly; in short term studies no effects are seen. This is illustrated in Figure 9, in which concentrations of saturated, mono- and diunsaturated fatty acid groups are indicated by bars. After ten weeks on a formula containing forty per cent of calories as corn oil, the adipose tissue of this subject was normal in fatty acid composition. Then, after thirty-eight days on a fat-free high carbohydrate formula, the adipose pattern was still unaffected. During ten weeks on high intakes of corn oil, this patient ingested more than 3.5 kg. of linoleic acid, an amount roughly three times that present in his entire adipose tissue. Yet, the adipose linoleic concentration did not rise. During thirty-eight fat-free days the patient's caloric expenditure was of the same order of magnitude as the total caloric value of his entire adipose depot, yet body weight did not change nor was adipose composition altered. Clearly, there must have been intense lipogenesis from carbohydrate; but, the lack of accumulation of 16:0 and 16:1 and lack of decrease in 18:2 strongly argues against the participation of the entire adipose depot in the new synthesis of fatty acids.

When formulas rich in corn oil are fed over extremely long periods, slow changes in adipose composition are seen eventually, and they continue until an adipose pattern is evolved which is very similar to that of the fed corn oil acids. Figure 10 is a composite of the adipose changes in eight different subjects receiving diets rich in corn oil (usually 40 per cent of calories) from eight to 160 weeks. Changes are almost imperceptible up to twenty weeks. However, at 160 weeks the adipose tissue has become rich in diunsaturated acid (essentially all 18:2) and it closely resembles a mixture of corn oil and normal adipose triglyceride in proportions of 7 to 3. There is no reason to believe that even further adipose changes might not have occurred if the corn oil feedings had been continued for longer periods of time.

COMMENTS

This technic, whereby numerous samples of human adipose tissue can be analyzed rapidly and accurately, opens many investigative pathways. Only a few of these have been explored

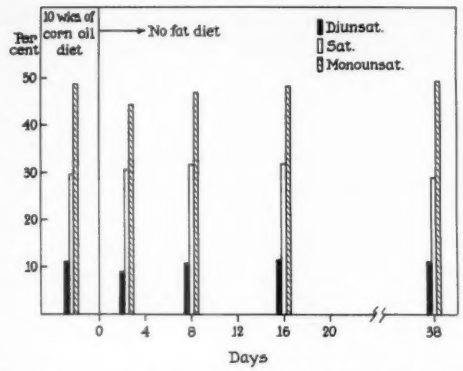

FIG. 9. The effects of fat-free diet on the composition of adipose tissue in terms of diunsaturated (18:2), monounsaturated (16:1 and 18:1) and saturated (14:0, 16:0 and 18:0) acids. These findings supplement those of Figure 8.

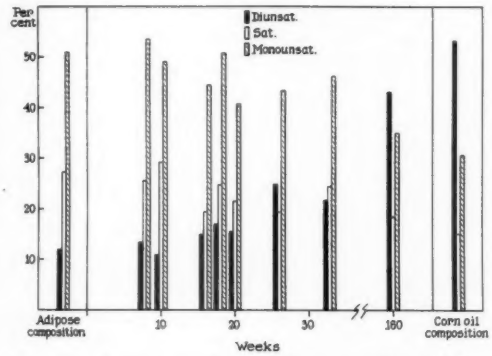

FIG. 10. Long term dietary effects on adipose tissue. The adipose compositions of eight patients on diets rich in corn oil (usually 40 per cent of calories) are compared with the normal adipose composition as well as with the fatty acid structure of corn oil. For abbreviations, see legend to Figure 9.

in a preliminary way in this paper, and thus it is too soon to assess with any finality the many forces which create specific fatty acid compositions in adipose tissue. Obviously, there are broad similarities to the well known early investigations of "hard" and "soft" animal fats. The classic work of Mendel and Anderson in this area, reviewed nearly thirty years ago,[20] might have predicted many of the present findings. But, one aspect of the present study and of the older animal work as well remains particularly puzzling.

If one considers the rate at which dietary fat

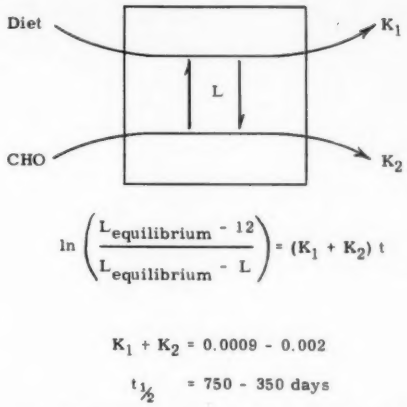

FIG. 11. Kinetics of adipose fatty acids. Adipose tissue is represented as a single pool of completely miscible fatty acids of constant size with linoleic acid concentration = L. L is being turned over under two influences, at least: (1), mixing with dietary fat (K_1); and (2) new synthesis of fatty acids from carbohydrate coupled with removal of acids for energy (K_2). When a diet rich in linoleic acid is fed, there is a slow increase in L from 12 (normal content at start) to $L_{equilibrium}$. In estimating the $t_{1/2}$ from the data of Figure 10, $L_{equilibrium}$ has been considered to lie somewhere between 44 per cent (the observed L in one patient after 160 weeks of high corn oil intake) and 53.5 per cent (a theoretical maximum equal to the 18:2 content of corn oil). Using several points from Figure 10 for L and t, $(K_1 + K_2)$ is found to range between 0.0009 and 0.002, hence $t_{1/2}$ is between 750 and 350 days.

exchanges with adipose fat, using Figure 10 as a guide, the exceedingly slow nature of this process is evident. One reasonable model of adipose fatty acid turnover is that of a large pool of acids being turned over by (1) the entrance of dietary fatty acids; (2) the synthesis of fatty acids *de novo* from carbohydrate; and (3) the exit of acids, most likely in the form of serum non-esterified fatty acids.[1] When dietary fat is suddenly enriched in linoleic acid, the rate at which a new equilibrium of linoleic acid content in adipose tissue is reached could indicate an over-all rate of fatty acid turnover in the depots, providing that all adipose fatty acids are readily miscible and can be treated as a single pool. Using the data of Figure 10 for this calculation, one arrives at the extraordinarily slow half-life of 350 to 750 days. Figure 11 shows such a calculation. When different points from Figure 10 are used,

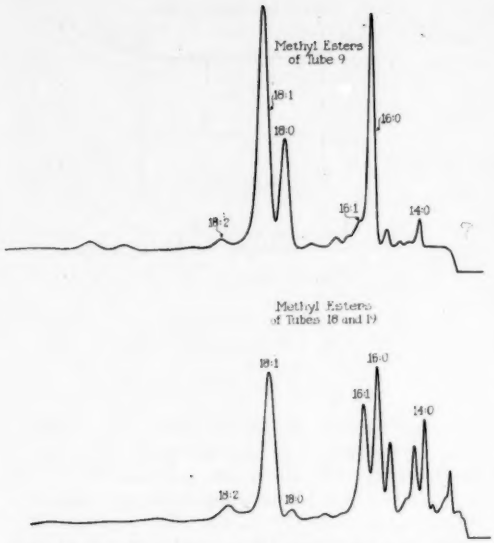

FIG. 12. GLC analyses of triglyceride fatty acids removed from different portions of the triglyceride elution curve shown in Figure 2. The presence of at least two completely different "species" of triglycerides is evident. Stationary phase = ethylene glycol adipate polyester, temperature = 185° C.

$(K_1 + K_2)$ ranges between 0.0009 and 0.0002, and the $t_{1/2}$ is therefore between 750 and 350 days.

However, numerous isotope studies have demonstrated that the turnover of depot fatty acids is far more rapid than these calculations indicate. Rittenberg and Schoenheimer[21] injected and fed mice with deuterated water and showed that depot fatty acids gain deuterium from body water in a curve reaching the midpoint at only five to nine days. More recently, Pihl, Bloch and Anker[22] demonstrated that labeled acetate was quickly incorporated into carcass fat of the adult rat with a half-life of sixteen to seventeen days and into the unsaturated acids of depot fat with a half-life of nineteen to twenty days. Furthermore, many recent studies of different types provide abundant evidence for brisk metabolic activity in adipose tissue.

One reconciliation of these contradictory approaches to the problem of depot fatty acid turnover is to postulate the existence of at least two separate metabolic compartments in adipose fat. If ready exchange of fatty acids between these pools does not occur, one basic assumption for the calculations of Figure 11 is not valid. The much larger compartment might serve as an inert storage for fat calories, exchanging only slowly with dietary fat. The much smaller and rapidly turning over compartment may be in close metabolic relation to dietary, serum and liver lipids. This smaller pool may also be the site of fatty acid synthesis from carbohydrate. Such compartmentalization could occur on a cellular, subcellular or even molecular level. The small cytoplasmic portion of adipose cells, or even the organelles within the cytoplasm, might contain special fatty acids both in the chemical and metabolic sense. Indeed, the different fatty acid positions (α and β) on the triglyceride molecule are not equivalent: lipases hydrolyze the α-ester linkages preferentially,[23] and furthermore, there are specific arrangements of saturated and unsaturated fatty acids at the α- and β-positions in most natural fats.[24] Thus, a possibility of intramolecular compartmentalization also exists.

It is certainly true that adipose fatty acids are organized into different species of triglyceride within adipose tissue. These variations between triglycerides become evident in preliminary separations by silicic acid chromatography. Figure 12 shows analyses of methyl esters prepared from the early and late portions of the triglyceride curve of Figure 2. In tube 9 the triglyceride is rich in 16:0, 18:0 and 18:1, but in tubes 18 and 19 a completely different array of shorter and more polar fatty acids occurs. Are these different species of triglycerides representative of one or another functional compartment of adipose tissue? Newer technics of triglyceride separation now under study in this laboratory may be informative in application to this problem.[25]

The possibility that the bulk of adipose fat is an inert pool of stored calories representing a sample of dietary fat integrated over many years makes the technic of adipose aspiration particularly attractive from the standpoint of nutritional epidemiology. If definitive proof of this idea is obtained, the technic may supplement and lend greater accuracy to nutritional surveys.

SUMMARY

A method is described for simple, virtually painless sampling of adipose tissue in man. Such samples (0 to 34.3 mg. in size) have been obtained from 145 subjects and analyzed for fatty acid composition by gas-liquid chromatography. In this way, a typical or "normal" pattern of adipose fatty acid composition in healthy, young adults has been established. Small groups of older, healthy persons as well as those with coronary artery disease, obesity and diabetes have shown no marked differences from the average pattern of healthy younger subjects. But, newly born premature and term infants have distinctive alterations. Furthermore, controlled dietary manipulations in adult life can give rise to slow changes in adipose composition. The possible significance of these findings is discussed.

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Clinical Report

Co^{60}-Vitamin B_{12} Binding by Chromatographic Fractions of Human Gastric Contents

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SUBSTANCES present in the gastric juices of several species bind vitamin B_{12}, making the vitamin non-dialyzable and unavailable to certain microorganisms. The possibility that the phenomenon of vitamin B_{12} binding might be correlated with intrinsic factor activity has interested many investigators. Ternberg and Eakin[1] were the first to suggest this relationship when, by using a bacteriologic assay, they found that gastric juice from normal human subjects bound more vitamin B_{12} than did gastric juice from patients with pernicious anemia. While this work has been accepted generally, little investigation has been carried out to prove whether or not the near absence of binding found by these investigators is a consistent result in patients with pernicious anemia. On the other hand, this binding phenomenon cannot be considered completely synonymous with intrinsic factor activity since there are many binding substances which fail to correct the absorptive defect associated with pernicious anemia. Heating gastric juice will destroy intrinsic factor, but not all of its binding ability,[2] and some potent sources of hog intrinsic factor have been found to bind vitamin B_{12} poorly.[3] Whether vitamin B_{12} binding is essential to intrinsic factor activity is not yet proved.

The binding substances in human gastric juice have been separated by several investigators. Using barbital buffer at pH 8.6, Schilling and Deiss[4] were able to separate one anodal migrating binding peak. Using a similar buffer system, but a different binding assay, Latner and Ungley[5] observed two binding peaks at pH 8.6 and at least seven binding peaks at pH 6.35. Only one major binding peak was noted by Grasbeck,[6] who thinks that the multiple binding peaks noted by other investigators may be due to autodigestion.

Richmond et al.[7] recently described a resin chromatography method for fractionating human gastric juice. The effluent from these columns is ideal for measuring vitamin B_{12} binding, since several discrete fractions can be obtained, relatively large quantities of each fraction are available for analysis and the effluent is known to contain intrinsic factor.[8] We have used a modification of this technic for individual samples of human gastric juice in an attempt to clarify the relationship of binding and intrinsic factor activity.

PROCEDURE

Individual samples of human gastric contents were dialyzed, lyophilized and redissolved in citric acid buffer (pH 3.1) or in citric acid buffer containing Co^{60}-labeled vitamin B_{12} in a concentration of 0.01 μg. vitamin B_{12} per mg. of gastric juice. This mixture was allowed to dissolve overnight in the refrigerator. The sample was centrifuged and 1 ml. of the supernatant (containing 30 mg. of dissolved gastric juice or gastric juice and radioactive vitamin B_{12}) was introduced onto an Amberlite IRC-50

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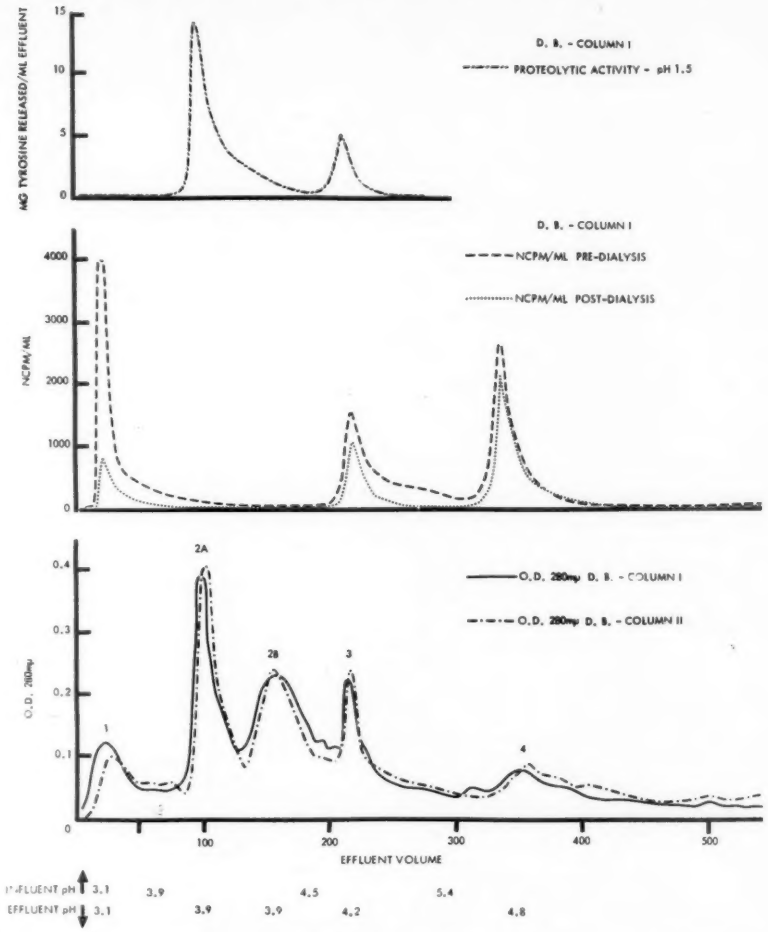

FIG. 1. Case 1. Chromatographic pattern of a patient with peptic ulcer. Liver uptake of Co^{60}-labeled vitamin B_{12} was normal.

resin column. The column was 10 cm. long, 1.5 cm. in diameter and was previously equilibrated to pH 3.1 with citric acid buffer.

The gastric material was fractionated by stepwise increases in pH (3.1, 3.9, 4.5, 5.4 and 6.1) by the addition of citric acid buffers. Fractions were collected in approximately 5 ml. volumes with a flow rate varying from 20 to 24 ml. per hour. The total running time was about forty-eight hours.

Absorption at 280 μ was used as an indication of the amount of protein material present. Carbohydrate was measured by the anthrone reaction[9] and pepsin by the method of Anson and Mirsky. The "tyrosin-like" substances which resulted from digestion were measured spectrophotometrically at 280 μ. When gastric juice was fractionated with Co^{60}-labeled vitamin B_{12} the radioactivity of each fraction was determined. These fractions were then dialyzed in cellulose tubing against continuously running tap water (pH 9) for twelve hours and mechanically agitated against distilled water (pH 5.5) for twelve hours at 10°C. The amount of radioactivity was again determined. Some samples were dialyzed against citric acid (0.2 M) and phosphate (0.15 M) buffers for forty-eight hours at 10°C. Bound

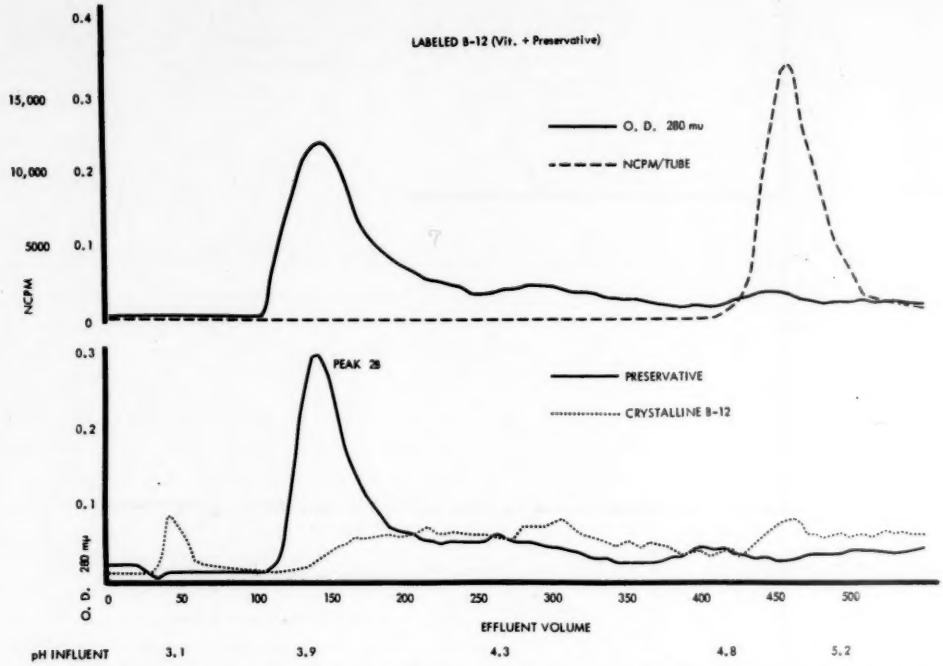

FIG. 2. Chromatographic patterns of crystalline vitamin B_{12}, commercial radioactive vitamin B_{12} and the preservative present in radioactive vitamin B_{12}. The preservative gives a sharp peak at 3.9 (area of peak 2B, Fig. 1). Radioactive vitamin B_{12} appears as a sharp peak at effluent pH 4.8. The optical density present with crystalline vitamin B_{12} is due to breakdown of the resin.

vitamin B_{12} was considered to be that portion of the Co^{60}-vitamin B_{12} remaining in the dialysis sac.

Gastric juice from four patients was chromatographed. One patient (Case 1), a forty-two year old man, had a clinically inactive duodenal ulcer but no clinical evidence of pernicious anemia. Liver uptake of Co^{60}-labeled vitamin B_{12} was within normal limits.[11] Another patient (Case 2) had chronic urticaria but no evidence of pernicious anemia. A twenty-nine year old man (Case 3) had diabetes of less than one year's duration. There was no evidence of pernicious anemia and Co^{60}-vitamin B_{12} liver uptake was normal.[11] The gastric juice contained no free acid or pepsin but did contain clinically active intrinsic factor when tested in a patient with pernicious anemia. A sixty-five year old woman (Case 4) had pernicious anemia in remission. When this patient was first seen, marked anemia, megaloblastic bone marrow, gastric anacidity and a peripheral neuropathy were present. Liver uptake of vitamin B_{12} was 1.5 per cent and increased to 3.3 per cent with the simultaneous ingestion of human gastric juice.[11] Anemia and peripheral neuropathy responded completely to vitamin B_{12} administered intramuscularly. Gastric content of this patient did not contain pepsin or intrinsic factor activity when tested in another patient with pernicious anemia. Fasting gastric contents were obtained from each patient following the intravenous injection of 10 units of regular insulin.

Co^{60}-labeled vitamin B_{12} from Merck and Company, Inc. and its preservatives, 0.15 per cent methylparaben and 0.02 per cent propylparaben, were chromatographed at the same concentration as was present in the gastric juice.

RESULTS

Figure 1 shows the chromatographic pattern of gastric juice obtained from a patient with

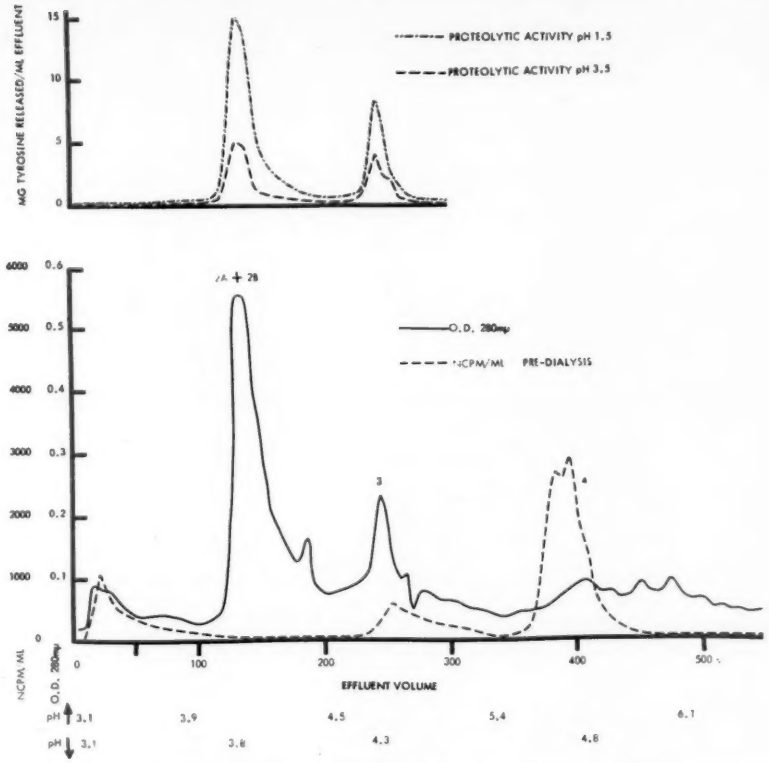

FIG. 3. Case 2. Chromatographic pattern of a patient with chronic urticaria. Liver uptake of Co^{60}-labeled vitamin B_{12} was normal.

peptic ulcer (Case 1). Duplicate columns are plotted. The effluent shows four protein peaks at pH 3.1, 3.9, 4.2 and 4.8. The peak at pH 3.1 was the only anthrone positive peak and showed blood type "A" substance activity. The first peak at pH 3.9 (P-2A) and the peak at 4.2 (P-3) contained proteolytic activity. The peak at 4.8 is of unknown physiologic activity. Three Co^{60}-vitamin B_{12} binding peaks are present at pH 3.1, 4.2 and 4.8.

The second peak released at pH 3.9, P-2B, is identified (Fig. 2) as the preservatives methyl- and propylparaben, present in commercial radioactive vitamin B_{12}. The labeled vitamin B_{12} appeared in the area of peak 4 and was dialyzable. When the preservatives were chromatographed alone, they appeared in the effluent at pH 3.9 and were dialyzable. Due to the low concentration, crystalline vitamin B_{12} showed no optical density at 280 μ.

In Case 2, four protein peaks can be readily recognized (Fig. 3) and are numbered in the order in which they appeared in the effluent. Proteolytic activity was present in peaks 2A and 3. In this chromatogram no clearly defined peak appeared in the area between peaks 2 and 3. Peak 2B was separated from peak 2A in the previous chromatogram by placing the gastric juice on the column in a narrower band. In addition to the protein pattern, the concentration of Co^{60}-labeled vitamin B_{12} is plotted as it appeared in the effluent. The radioactivity appeared in peaks 1, 3 and 4. After dialysis, radioactivity was still present in each of the peaks with the greatest percentage present in peak 4. Proteolytic activity determined at pH 3.5 and 1.5 is also plotted.

Figure 4 shows the results obtained after proteolytic inactivation of normal gastric juice from a patient (Case 2) by the method of

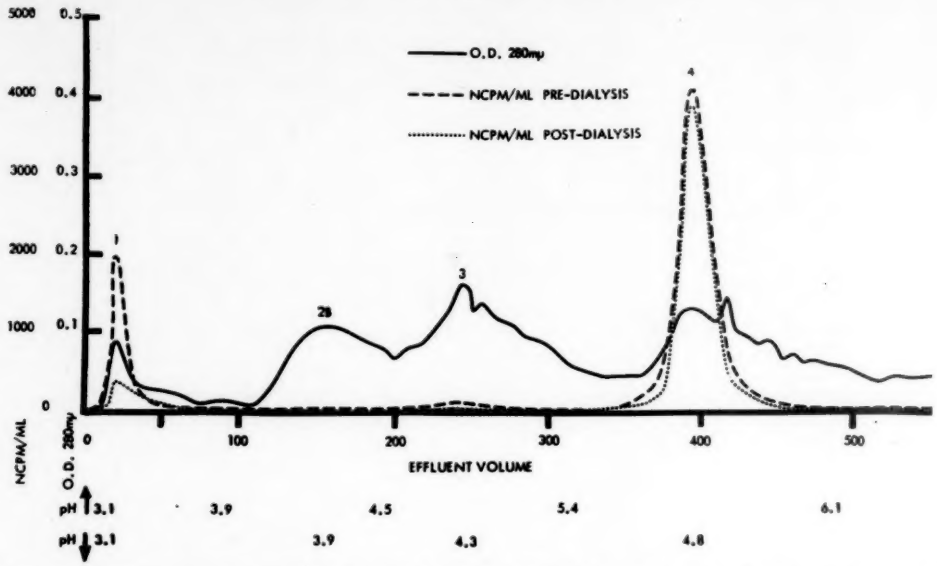

FIG. 4. Case 2. Chromatographic pattern of a patient with chronic urticaria, after inactivation of proteolytic activity.

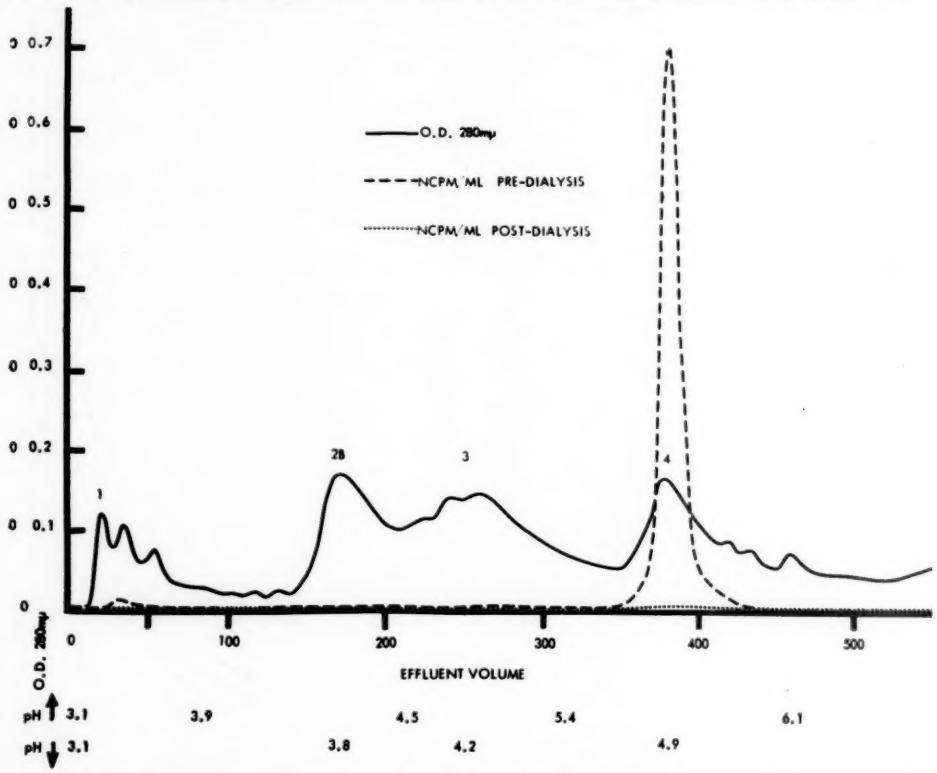

FIG. 5. Case 3. Chromatographic pattern of a patient with diabetes mellitus. Liver uptake of vitamin B_{12} was normal.

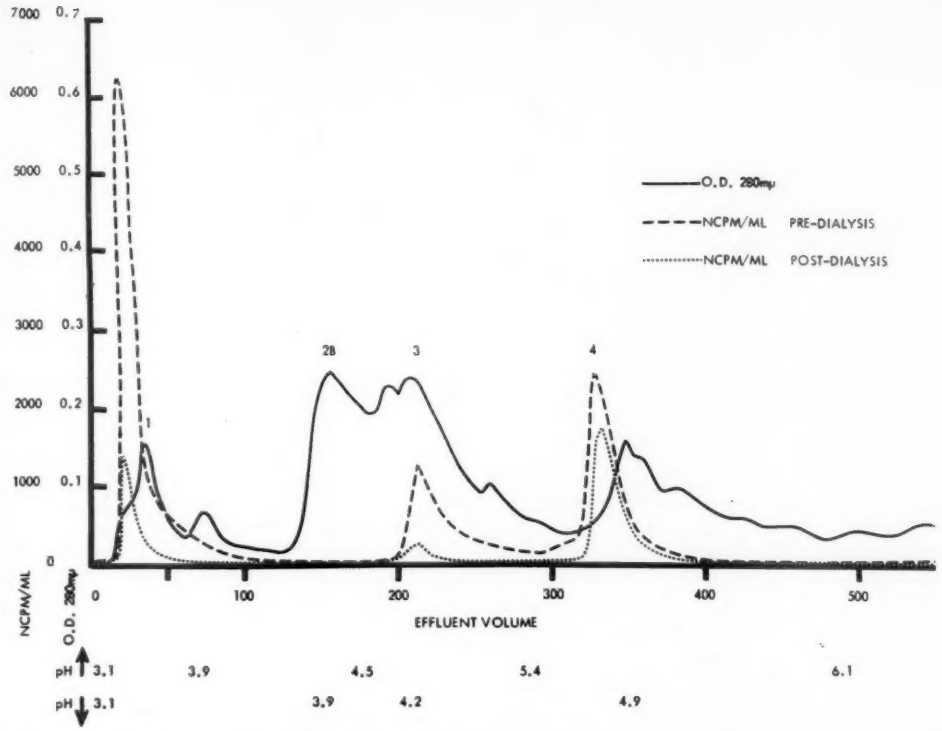

FIG. 6. Case 4. Chromatographic pattern of a patient with pernicious anemia, partially characterized by insufficient liver uptake of vitamin B_{12}.

Grasbeck.[6] This destroyed peak 2A and the peptic activity of peaks 2A and 3. Vitamin B_{12} was present in the effluent in peaks 1 and 4. After dialysis, most of the radioactivity remained in each peak.[4]

Figure 5 is the gastric juice chromatogram of a patient (Case 3) with diabetes. This gastric content did not contain peptic activity and peak 2A is absent. As in Figure 2, Co^{60}-labeled vitamin B_{12} appeared in the chromatographic pattern at pH 4.9 and almost all the radioactivity was dialyzable, showing that protein binding was virtually absent.

Figure 6 represents the chromatographic pattern of another patient (Case 4) with pernicious anemia. All protein peaks are present except for pepsin peak 2A. No peptic activity was present in this gastric content. The binding pattern is quite similar to that obtained from the normal patients before and after dialysis.

Table 1 shows the vitamin B_{12} bound by each peak of various gastric juices. The second subject (Case 2) demonstrated more binding of vitamin B_{12} in peak 4 than did the first patient. This is probably the result of individual variation. The proteolytic activity of the gastric juice obtained from this patient (Case 2) was

TABLE 1
Comparison of Vitamin B_{12} Binding by Various Gastric Juice Fractions

Case No.	Condition	Peak No. (μg. vitamin B_{12}/fraction)		
		1	3	4
1	Peptic ulcer	24	30	67
2	Chronic urticaria	20	7	133
2	Inactivated	7	<1	153
3	Diabetes mellitus	1	0	1
4	Pernicious anemia	30	11	63

TABLE II

Effect of pH of Dialysis Bath on Vitamin B_{12} Binding of Unfractionated Gastric Juice from Normal Subjects and Patients with Pernicious Anemia

Subject	μg. Vitamin B_{12} Bound/mg. Gastric Juice			
	Distilled Water	Citric Acid Buffer 0.2 M		
		pH 3	pH 4	pH 7
Normal (Case 2)	8.2	..	..	..
Normal	8.9	9.0	8.6	9.0
Pernicious anemia	7.1	6.8	7.1	7.5
Pernicious anemia	8.7	8.5	8.5	8.1
Pernicious anemia	9.6	9.2	9.4	10.0
Pernicious anemia (Case 4)	8.7	8.2	8.5	8.4

NOTE: Initial concentration of 10 μg. vitamin B_{12}/mg. gastric juice.

destroyed and the change in binding is shown. Changes occurred in each binding peak with peak 4 binding more vitamin B_{12} and peaks 1 and 3 binding less. Shown next is the gastric juice of a patient with diabetes (Case 3) but no vitamin B_{12} absorptive defect. Last is a patient (Case 4) who had pernicious anemia. Since the gastric juice from the patient with pernicious anemia bound vitamin B_{12} normally we measured the binding ability of the unfractionated gastric juice of three other patients with pernicious anemia (Table II).

The vitamin B_{12} binding of gastric juices of normal subjects and patients with pernicious anemia was also measured dialyzing against citric acid buffer pH 3, 4 and 7 (Table II).

TABLE IV

Effect of Vitamin B_{12} Concentration on Binding μg. Vitamin B_{12}/mg. Gastric Juice

Amount of Vitamin B_{12} Available	Amount of Vitamin B_{12} Bound		
	Normal Subject	Patient with Pernicious Anemia	Patient with Pernicious Anemia
2.5	2.1	2.1	2.5
5	4.0	4.3	3.9
10	8.2	8.1	8.6
15	12.0	12.4	13.6
20	12.3	15.6	14.0

Grasbeck reported that vitamin B_{12} binding by intrinsic factor active fraction of gastric juice was destroyed by dialyzing against buffer pH 3, 4 but not at pH 7.[8] The effect of the pH of the dialysis bath on vitamin B_{12} binding by chromatographic fractions of normal gastric juice using buffers at pH 3, 4, 4.8 and 7, is shown in Table III.

The vitamin B_{12} binding by normal gastric juice buffered at pH 1, 1.5, 2, 2.5, 8 with citric acid and phosphate buffers prior to the addition of vitamin B_{12} was measured. The presence of these buffers had no effect on the vitamin B_{12} binding ability of this gastric juice.

Using a microbiologic method, Raine has reported that the binding of intrinsic factor and vitamin B_{12} varied directly with concentration of the vitamin.[12] Table IV shows the effect of vitamin B_{12} concentration on the binding ability of the gastric juice taken from a normal subject and two patients with pernicious anemia. This table shows that binding

TABLE III

Effect of pH of Dialysis on Vitamin B_{12} Binding by Normal Gastric Juice Fractions

Peak No.	Effluent pH	μg. Vitamin B_{12} Bound					
		Distilled Water	Tap Water	Citric Acid Buffer 0.2 M			
				pH 3	pH 4	pH 4.8	pH 7
1	3.0	14.9	14.8	14.2	16.3	..	14.5
3	3.8	6.0	6.1	..	6.0	..	..
4	4.8	6.9	7.0	7.1	7.0	6.7	6.8

NOTE: Concentration of vitamin B_{12} placed on column = 10 μg. vitamin B_{12}/mg. gastric juice.

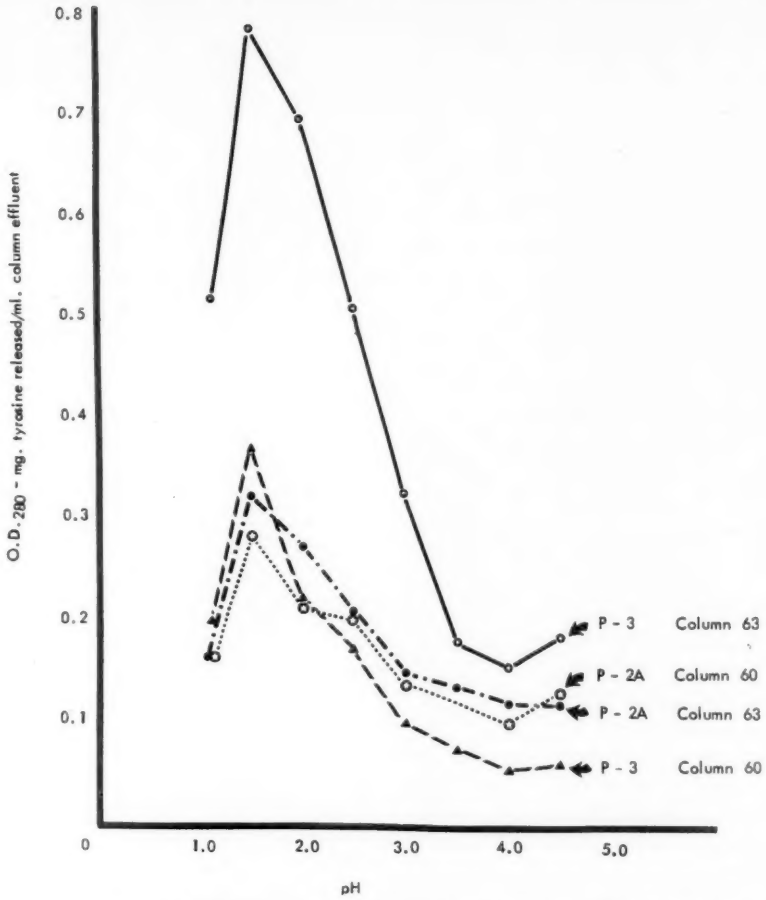

FIG. 7. Proteolytic pH activity curve—hemoglobin substrate. Effect of pH on the proteolytic activity of the two chromatographic peaks which showed proteolytic activity. Gastric juice was obtained from two normal volunteer subjects.

varies linearly with concentration of vitamin B_{12}.

Proteolytic activity appears in two more or less sharply defined peaks in the effluent of this fractionation. The first proteolytic peak appears at effluent pH 3.9 and the second peak at pH 4.2. One of these peaks binds vitamin B_{12}. This would suggest that the two proteolytic peaks might represent two different enzymes. In an attempt to clarify this point, the effect of pH on the proteolytic activity was measured (Fig. 7). The proteolytic peaks from the gastric juice of two different normal volunteer subjects were used. Both proteolytic peaks show a pH optimum of 1.5 which is characteristic of pepsin.

COMMENTS

When dialized and lyophilized human gastric juice is chromatographed on an IRC-50 resin column from pH 3.1 to 6.1, four more or less well defined protein peaks appear in the effluent. Three of these protein peaks are capable of binding to vitamin in B_{12} and in this way make the vitamin non-dializable. This binding is not pH sensitive, but is related to the concentration of vitamin B_{12} added prior to dialysis.

The first of these binding peaks appearing in the effluent at pH 3.1 is associated with most of the mucopolysaccharides present in the human gastric juice. This protein peak also contains A substance activity.[7,8] The second vitamin B_{12} binding peak appears at pH 4.2 and it is associated with one of the two peaks which have proteolytic activity. A third vitamin B_{12} binding peak is found in a protein peak which appears at pH 4.8. No known physiological activity has as yet been associated with this peak. Inactivating peptic proteolytic activity prior to chromatography of the human gastric juice reduces the binding of vitamin B_{12} by the first two protein peaks, the binding of the proteolytic peak being virtually destroyed. However, it does not change the binding activity of the third binding peak which appears in the effluent at pH 4.8.

Grasbeck, using starch column electrophoresis at pH 7.4, was able to isolate one major vitamin B_{12} binding peak from human gastric juice in which the peptic activity had been destroyed prior to electrophoresis.[6] In addition, two minor binding peaks seem to be present in the diagrams of this author. Grasbeck noted that if proteolytic activity was not immediately inactivated after collection, one of these minor binding peaks increased markedly in its ability to bind vitamin B_{12}, which was presumed to be due to autodigestion. The results presented here suggest that the inactivation procedure iteslf may change binding activity.

Glucosamine and hexuronic acid were present in high concentrations in the major binding peak. On the other hand, carbohydrate was present only in low concentrations in the major protein peak. It would appear that the major vitamin B_{12} binding peak noted by Grasbeck most closely corresponds to the first peak in our chromatographic fractionation, since both contain carbohydrate. In that study, gastric juice from normal human volunteer subjects was used and no patterns were shown of the gastric juice from patients with pernicious anemia.[6]

Using filter paper electrophoresis, Latner et al. detected two vitamin B_{12} binding peaks when gastric juice and radioactive vitamin B_{12} were fractionated at pH 8.6.[5] When large pools of gastric juice were used several vitamin B_{12} binding peaks were evident after electrophoresis at pH 6.35. At least two of these vitamin B_{12} binding peaks contained intrinsic factor activity. No comment was made as to the presence or absence of proteolytic activity in these binding peaks. The gastric juice from patients having pernicious anemia was not chromatographed. Schilling and Deiss,[4] using paper electrophoresis at pH 8.6, were able to find at least one anodally migrating vitamin B_{12} binding peak which, as in the work of Grasbeck, was distinct from the major protein components. In these experiments, the vitamin B_{12} was added to the gastric juice prior to the electrophoretic separation.

The protein peaks from the effluent of our columns were tested for intrinsic factor-like activity by the use of the rat liver slice technic.[13] These results showed that each of the binding peaks and also, to a lesser degree, the nonbinding protein peak, demonstrated intrinsic factor-like activity when used in the proper concentration with the rat liver slice. We have also tested these binding peaks for intrinsic factor activity by giving them with Co^{60}-labeled vitamin B_{12} to patients with pernicious anemia. Sufficient data have not been obtained as yet to make a conclusive statement. However, preliminary data would suggest that each of the binding peaks demonstrate some intrinsic factor activity at the proper concentrations. On the other hand, the protein peaks obtained from the chromatographic fractionation of gastric juice from patients with pernicious anemia failed to show intrinsic factor-like activity in the rat liver slice technic. These fractions also failed to enhance absorption of vitamin B_{12} when given orally with radioactive vitamin B_{12} to patients with pernicious anemia. This would suggest, then, that intrinsic factor is distributed throughout the effluent of these columns and not necessarily associated with the protein peaks which show vitamin B_{12} binding.

The data presented here show that under these conditions the gastric juice of patients with pernicious anemia is capable of binding vitamin B_{12} normally. This is different from

the results of others who used bacteriologic assays for measuring binding.[14,15] They found that the gastric juice from patients with pernicious anemia bound less vitamin B_{12} than did gastric juice from normal subjects.

The dialysis methods and the bacteriologic methods for measuring vitamin B_{12} binding to gastric juice have been found to give different or similar results when they were performed simultaneously. The bacteriologic assay of binding can be performed in two general ways. In the original method, the gastric juice and vitamin B_{12} were added directly to the culture media.[1, 16] In the other method, gastric juice and vitamin B_{12} are subjected to ultrafiltration and the ultrafiltrate is added to the media.[17] The ultrafiltrate method will give results similar to the dialysis method.[17] To our knowledge, no one has compared the binding ability of human gastric juice taken from normal subjects and patients with pernicious anemia by the dialysis method used in the present study. Since patients with pernicious anemia produce considerably less gastric juice than do normal subjects, the total amount of binding substances produced per day by a patient with pernicious anemia would be vastly less than that produced by normal persons. If vitamin B_{12} binding is a prerequisite to efficient absorption of vitamin B_{12} from the gastrointestinal tract, then it is possible that patients with pernicious anemia lack sufficient amounts of the binding substances to promote optimal vitamin B_{12} absorption. Much to our surprise, gastric juice from the subject with diabetes failed to demonstrate vitamin B_{12} binding even though symptoms of pernicious anemia were not present. In addition, the gastric juice lacked peptic activity. However, gastric juice from this patient (Case 3) was capable of correcting the absorptive defect of the patient with pernicious anemia. This patient with diabetes demonstrated normal vitamin B_{12} absorption as measured by liver counting after the ingestion of Co^{60}-labeled vitamin B_{12}. It would suggest then that, at least in this patient, vitamin B_{12} binding and peptic enzymatic activity are not a prerequisite to intrinsic factor activity. In addition, it would suggest that intrinsic factor is produced by mechanisms which are different from the production of the binding substances and the production of the peptic enzymatic activity.

Since vitamin B_{12} binding is present in gastric juices from patients with pernicious anemia and since normal vitamin B_{12} absorption and intrinsic factor activity can be present without binding, one might ask whether non-specific binding of vitamin B_{12} by gastric protein actually hinders vitamin B_{12} absorption. Binding does hinder absorption of vitamin B_{12} by certain bacteria and this fact is used in the assay of binding activity. Since vitamin B_{12} is freely dialyzable, yet exists in the gastrointestinal tract in the bound form, it is possible that intrinsic activity is necessary to break down this combination of gastric protein and vitamin B_{12} prior to absorption.

Using the dialysis method, we found that in the range tested pH did not change the binding of vitamin B_{12} by whole gastric juice and by fractions of gastric juice. These results confirm the work of other investigators, who found that pH changes did not alter the binding capacity of whole human gastric juice. The failure to find a change in binding of gastric factions at these pH's is different from the work of Grasbeck.[4] However, since Grasbeck measured binding by a biological method, his results may not be directly comparable.

The results presented here, using dialysis as a method for measuring vitamin B_{12} binding, are different in several respects from the data obtained by others using microbiological assays. These obvious differences would seem to make unsafe any conclusion of physiologic significance based on these binding data.

CONCLUSIONS

Gastric contents of normal subjects and patients with pernicious anemia and fractions of these gastric contents demonstrated equal affinity for making vitamin B_{12} non-dialyzable.

Gastric content without peptic activity from a patient with diabetes did not make vitamin B_{12} non-dialyzable, yet this gastric content showed intrinsic factor activity.

Inactivation of peptic activity changes the binding of vitamin B_{12} by fractions of human gastric contents.

Vitamin B_{12} binding as measured by dialysis is not affected by pH changes from 3 to 7 but is affected by the concentration of vitamin B_{12}.

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Diet Therapy

Interviewing the Patient

CHARLOTTE M. YOUNG, PH.D.*

THE ABILITY to do a good job of interviewing patients, whether for medical, social or dietary purposes, can be an extremely valuable asset. Often the key to a diagnosis may be in what the patient tells you if he is given the opportunity under circumstances in which he feels comfortable. One of the keenest diagnosticians I have ever known when asked how he arrived at diagnoses others had missed often replied, "Why the patient told me."

Good dietary interviewing, too, can be a valuable diagnostic aid. The ability to obtain a reliable dietary history and thus appraise the dietary status of the patient and its contribution to his total nutrition may be the best way to obtain an early clue to potential nutritional difficulties. It also forms the basis for the individual instruction of the patient when special dietary treatment seems indicated.

Good dietary interviewing requires skill, time and some background knowledge of what goes into forming food habits, their significance and the factors which affect them. If dietary history or dietary instruction is worth doing at all, it is worth doing well. This article is addressed to those who believe that the diet of the individual and his nutrition are a matter of concern to his total health.

It is important to be aware that food intake and eating habits represent one of the most complex facets of human behavior. When one attempts to influence food habits he is not dealing just with physical nourishment or an intellectual matter. One is using instead an intellectual approach to a highly involved behavior pattern. Wallen[1] has said that feeding activity ranks with sexual behavior as a demonstration of that peculiar and delicate interaction of biological, psychological and cultural influences so often found in the study of human wants.

Acceptance of food is a composite of biochemical, physiologic, psychologic, sociologic, cultural and educational factors. When this is understood and appreciated one can readily understand why it is one of the most difficult aspects of human behavior to change. One can also understand why a good dietary interviewer needs to learn all he can about the person being interviewed. People cling to their customary food habits, especially when other disquieting events may be taking place in their lives, for food has far more significance to them than physical nourishment alone. This realization may cause the physician to question whether the advantage to be gained by the special diet is worth the disruption of the security the patient has in his present patterns. However, once the decision has been made in favor of such disruption every effort must be made to fit the therapy to the patient and his immediate circumstances. One should not delude himself that giving a printed diet list to an unprepared patient is a form of therapy. Unfortunately, this is exactly what the busy physician thinks he must do: pull a printed diet list from the file that is proper therapeutically to the condition of the patient, tell the patient to follow it, and then believe he has discharged his responsibility. Actually poor

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diet therapy is often worse than no therapy at all for the patient may be switched from a "not too bad" normal regimen to one which he only partially understands and can only partially follow. If a therapeutic diet is worth prescribing, it is worth presenting in such a manner and adapting to the patient in such a fashion that there is some hope he may follow the diet. Occasionally, perhaps, the limited time spent partially presenting a therapeutic diet might better be spent in helping the patient to achieve a reasonably decent normal dietary regimen.

THE PHYSICIAN

Dietary interviewing takes skill, and is dependent upon good rapport between the patient and the interviewer. Not everyone has this ability. A good physician should be an excellent person for dietary interviewing since, in many cases, he is extremely skillful at dealing with the variables of human personality that are involved in the developing of rapport. In these cases the physician should interview and instruct the patient if he can possibly take the time and if he has a real interest in diet therapy. His prestige and status with the patient adds significantly to anything he has to tell the patient. In a study among homemakers in two big cities in New York State it was found that what the doctor had told them about food impressed them more than the information given them by any other professional person.[2] Such findings indicate an opportunity, challenge and obligation for the busy physician. Furthermore, since diet and food habits are so intimately connected with many aspects of a patient's life, a good diet interview may, in an indirect fashion, give the physician excellent clues to the general emotional state of the patient, his living conditions, etc., which could be of value in the therapy as a whole. This is another reason why it is particularly desirable for the physician to interview the patient if he has the time and interest.

If, however, the physician does not have the time, interest or particular skill to interview the patient he may turn this portion of his responsibility over to someone who does: a nutritionist, dietitian, nurse or even office helper if she is the right kind of person and has been instructed properly.

THE INTERVIEWER

The interviewer should be one who recognizes and understands the needs and attitudes of the patient, i.e., his need to protect or preserve his self-respect, which may be expressed as anger, hostility, defiance or "superior" attitude; his fear, expressed as insecurity, anxiety and the various means used to cover up fear; and his need for dependency and love.[3] The interviewer needs to be aware of the various needs and attitudes of the patient, to consider each patient as an individual, and to know what such attitudes mean if she is to be more understanding when dealing with the patient. She should have the ability to put the patient at ease in a warm friendly manner backed by absolute sincerity and honesty. Behind this must be a basically sincere interest and love of all kinds of people. A relaxed manner (sometimes hard to achieve when pushed for time) and a quiet unhurried "willingness to listen" create an easy atmosphere. It is a matter of building the right climate so that the patient will feel free to talk. Too many interviewers, in their own uneasiness, tend to chatter to fill the time lapses or, worse yet, jump to conclusions or put words into the patient's mouth. It is a matter of taking time and being willing to listen. Empathy, the ability to project one's self into the role of the patient with sensitivity to his needs and feelings but without over identification, is a great help. The interviewer must have an unjudging, unchastizing attitude with the ability to appear interested in but unsurprised by anything the patient may tell her. She must be capable of great flexibility, insight and practicality, always keeping in mind the real problems of the patient.

If the doctor does assign the dietary interviewing responsibility to someone else then he must give this person and her function prestige value in the eyes of the patient. He should make provision for keeping informed on what is going on and for receiving a report from the therapist or the patient. It is just as impor-

tant that the physician be aware of this aspect of therapy so that it can be tied in with the other therapy of the patient. Again we urge that no one tamper with the eating habits of the patient unless he intends to carry the therapy through and make it a constructive part of the total program.

TECHNICS

How one carries out the dietary interview depends upon the patient and the therapist. There are no uniform or standard procedures which should or could be laid down as applicable to all patients in the hands of all therapists. The method should be adjusted to the needs and conditions of the patient at the moment the instruction is being given and to the particular skills of the therapist.

One factor is certain, adequate dietary interviewing takes time. Little can be accomplished in most initial interviews during the ten or fifteen minutes often allotted. Sufficient time must be provided either through a longer initial interview or through closely timed follow-up interviews. Often there may be an advantage in the latter since initially the patient may be so confused and anxious that he absorbs far less than his intelligence might indicate.[4] If someone other than the physician does the interviewing she should introduce herself and make her role quite clear to the patient.

A climate should be built in which the patient feels relaxed and at ease so that he may talk more freely, remember better and receive instruction more easily. One wishes to know all he can about the patient: from his record, from other therapists and most especially from the patient himself. This can be accomplished by helping the patient to talk freely and then "reading between the lines." If the physician does the interviewing he probably has the advantage of an already established rapport as well as the knowledge of the background of the patient. Others may start by asking such general questions as height, weight, age, family composition, what he does, where he lives, etc. Often the extraneous conversation which these inquiries may produce (including either too profuse or too inhibited response) may be of more use than the answers to the specific questions in informing one about the patient. More specific questions with regard to food intake and habits may then be covered quite quickly: where he eats; with whom he eats; when and under what circumstances; who prepares the food; what preparation and storage facilities are available; shopping facilities; money available for food; the regularity of eating patterns; the usual (if any) eating pattern, as to what, amount and variations; between meal eating, what, when, under what circumstances; and, finally, a cross check of the information on actual food intake.

INDIVIDUALIZATION

Diet therapy should be built on the present food habits of the individual since these are already acceptable to him. Hence it is desirable to change them as little as possible. It has been said that the acceptability of food to the patient is the largest single factor in effecting changes in food habits. The advice given needs to be something which the patient can reasonably hope to adapt to his usual pattern of living. I believe a printed diet list should be avoided as much as possible since it is important for the patient to think that the plan is developed for him and his particular needs, as indeed it should be.

Dietary information should be summarized on the general clinic record of the patient and in addition, in detail on a card in a place set aside for it. Often this second record may contain a running report on subsequent visits as well which will help in re-establishing rapport and in finding the solution to continuing problems.

Dietary interviews usually should be followed by subsequent, perhaps briefer, visits. In certain cases too much information should not be given during the first interview for fear of overwhelming the patient in his anxious state. Often the patient can not absorb all the information given him during his first visit or he has no questions at the moment. However, when he tries to carry out the instructions he may find he has many questions. It is important that these be dealt with carefully

and not disregarded. Small practical points may make a difference in whether or not instructions will be followed. Also, the willingness of the interviewer to help on fine points may impress the patient with the importance of his diet to his general welfare. If the inquiry is brushed off, the patient interprets it as "can't be very important anyhow." If one does not know the answer to a question it is better to tell the patient so, to treat his question with respect and to try to find the answer. In many cases, it may be useful to talk not only with the patient but also with the person responsible for his food planning, purchase and preparation. If, during the dietary interview, one is primarily concerned with obtaining a reasonably accurate picture of present food intake, it may be advantageous in certain cases, to check the diet picture again later after the person has been sensitized to "being aware" of what he is eating. Most normal people, unless they have some food responsibilities, give little thought to their food habits, hence the value of the information may be greater after the person has been geared to think of what he eats.

Dietary interviews are best carried out in a quiet, calm place with some degree of privacy. This may exist in the office of the physician; however, if someone else is doing the interviewing it is particularly important to remember this need. Food habits are personal matters which deserve some privacy; also recall is much better when there is some freedom from distractions.

Any materials used in interviewing should be patient-centered. There is merit to brevity, simplicity of language, practicality, accuracy, logical sequence and factualness (which is what the patient is looking for when he actually begins to understand the material presented). Graphic presentation is particularly important if there are any language or numerical handicaps.

Interviewing can be rewarding if carried out with skill and interest and allowed sufficient time.

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